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The Ultrastructure of Rat Kupffer Cells
and the Phagocytosis of Colloidal Iron

Christina M. Trotter

Ph.D.
University of Edinburgh
July 1971
ACKNOWLEDGEMENTS

The author wishes to express sincere thanks to Professor A. R. Muir for his continual supervision and advice during this study and his criticism following the writing of the thesis. Thanks are also due to Dr. J. S. G. Cox for additional supervision and criticism and to Fisons Limited - Pharmaceutical Division for their generous financial support, supply of animals and ID.

Technical assistance from Mr. A. J. Murphy in the histology and Mr. N. Smith in the photography is gratefully acknowledged; Dr. M. Hines is thanked for his advice and assistance with atomic absorption spectrometry.

Lastly, Miss C. J. Garbutt is thanked for her co-operation and patience in the typing of this work.
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SUMMARY

This work follows from an interest in the total dose infusion method of administering iron to anaemic patients. A colloidal iron dextran (ID) of mean particle size 34 nm by 6 nm containing 200 mg Fe/ml was injected into 7-week-old healthy male rats giving single doses of 20, 60, 400 and 500 mg Fe/Kg body weight. Control rats were kept up to 59 wk and ID-treated were killed between 10 min and 1 yr after injection. Their livers were fixed by perfusion through the portal veins, the most successful method being with 1% OsO₄ in 0.17M sodium cacodylate at 0-4°C, pH 7.4 and at 8.8 mm Hg pressure.

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The tissue appeared healthy throughout the study, with the only differences between control and ID-treated being due to the high iron content in the cells.
1.0 Introduction

Many colloidal compounds are injected intravenously for therapeutic or experimental reasons, and these are removed from the plasma by phagocytic cells of the reticulo-endothelial system (RES) located mainly in the liver, spleen and bone marrow. One such compound is iron dextran (ID) which is used to treat iron-deficiency anaemia, and the total dose, sometimes a large amount of iron, may be infused at one time (Basu, 1963, 1965).

It is necessary, for safety, to know the cytological events following injection, as well as the long-term handling of the iron and its ultimate fate. When single high doses are administered it is particularly important to know how the body tolerates them. It is with these that the present work is concerned.

Colloidal iron dextran has many properties which make it a technically convenient tool for a study of phagocytosis. It is relatively non-toxic and can be given in doses two hundred times larger than any therapeutic dose. Its iron content can be identified by the
Prussian blue technique for light microscopy and one type (FPL 2000) has a characteristic fine structure which can be identified in even minute amounts in electron micrographs. ID is metabolised, so the cytological events following ingestion can be studied and the physiological iron stores of ferritin and haemosiderin can be structurally distinguished from the administered iron.

The following short reviews attempt to summarise the relevant knowledge of the structure of the hepatic sinusoid, the process of phagocytosis, the role of lysosomes and intracellular iron metabolism.

1.1 Structure of hepatic sinusoids

Hepatic parenchymal cells adhere to each other to surround bile canaliculi and so form anastomosing sheets, which are never more than two cells thick (Elias, 1955). These sheets are permeated by a labyrinth of vascular spaces connecting the hepatic portal vein and hepatic artery to the central or hepatic veins. The walls of these vascular spaces consist of a
thin cellular lining separated by a space of Disse from the vascular surfaces of the parenchymal cells.

1.1.1 Cellular lining

A vast, confusing and inconclusive literature has accumulated concerning the nature of the cellular lining of the sinusoids (Aterman, 1963). Certain points are not now in dispute; the lining is cellular, without a basement membrane in most species, and the cells show varying degrees of phagocytic activity. Most of the lining cells are very thin squames except in their perinuclear regions. The intercellular boundaries of these cells cannot be impregnated with silver nitrate as in other endothelia (von Kupffer, 1899) and some light microscopists such as Pfuhl (1926) and Wolf-Heidegger (1941) attribute this failure to stain intercellular boundaries to their absence. They conclude that the lining is syncytial, but the earliest electron microscopic study (Fawcett, 1955) shows its cellular construction and explains the failure to stain by showing that the usual intercellular junctional complexes such as zonulae occludentes,
adhaerentes and maculae adhaerentes
(Farquhar & Palade, 1963) are absent. Wisse (1970a) reports junctional complexes
characterised by a slight increase in electron
density of the membranes and neighbouring
cytoplasm. They may be discontinuous, with
gaps between them.

Most authors report that these lining
cells are discontinuous with gaps between them
ranging in width from 0.1-1 μm in rat
(Tanikawa, Yoshimura & Gohara, 1965) 0.1-0.3 μm
in rabbit (Yamagishi, 1959) 0.03-0.1 μm in
mouse (Schmidt, 1960) 0.04-0.35 μm in human
(Ito & Shibasaki, 1968). It is recognised
that these gaps could be shrinkage artefacts,
but at least they indicate a difference from
similarly treated endothelia in other tissues
(Fawcett, 1955). Wisse (1970a) is emphatic
that there are no gaps between cells, only
fenestrae within the endothelial cells, 0.1 μm
wide, and up to 0.25 μm apart, forming sieve
plates.

In the calf liver, Wood (1963) finds an
almost continuous lining with adjacent cell
membranes only separated by gaps of 20 nm. Similarly, only in the calf, Wood shows the constant presence of a basement membrane, although Burkel & Low (1966) report that, at the portal and central ends of the sinusoid in the rat, there are complete cellular linings supported on basement membranes which are continuous with those of the adjacent veins. So available reports indicate that, in animals other than the calf, the lining does not possess a basement membrane for over 90% of the length of the sinusoid (Burkel & Low, 1966; Rouiller, Pictet, Nicolescu & Orchi, 1967).

Table 1 summarises the features which are reported in the lining cells of hepatic sinusoids. Aterman (1963) reviews the early light microscopic work, which is now associated with the name of Carl von Kupffer (1876, 1899).

The main controversy regarding the lining cells is whether they belong to one or more cell types. Such differentiations could be demonstrated structurally or functionally, but if the former the changes must be distinct from those which would naturally follow from
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<tr>
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<tr>
<td></td>
<td>Rat</td>
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<td></td>
<td>Human</td>
<td>Wolf-Heidegger (1941)</td>
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<td></td>
<td>Frog</td>
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<tr>
<td>Stellate, with four processes</td>
<td>Fish, Reptiles, Birds, Mammals</td>
<td>Knisely, Bloch &amp; Warner (1948)</td>
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<td>Flat</td>
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<td>Pleomorphic</td>
<td>Birds, Mammals</td>
<td>Review</td>
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<tr>
<td></td>
<td>Fish, Reptiles, Birds, Mammals</td>
<td>Aterman (1963); Carr (1970)</td>
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<td></td>
<td>Review</td>
<td>Bouiller et al. (1967)</td>
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<td></td>
<td>Human, Rat</td>
<td>Tanikawa et al. (1965)</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Ito &amp; Shibasaki (1968)</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td></td>
</tr>
<tr>
<td><strong>Elongate, thin except in perinuclear region</strong></td>
<td>Fish, Reptiles, Birds, Mammals</td>
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<tr>
<td><strong>Size</strong></td>
<td></td>
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<td>14–16 μm long x 3–4 μm thick</td>
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<td>60–100 nm thick at nucleus, 25 nm elsewhere</td>
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<td>50–300 nm thick</td>
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<td>200–800 nm thick</td>
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<tr>
<td>----------------------------------</td>
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<td>Cell Surface</td>
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<td></td>
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<td>Human, Sheep, Fish, Reptiles, Birds, Mammals</td>
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<td>Rabbit</td>
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Cytoplasm
Impregnates with gold chloride
Granular and acidophilic
Granular
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<tr>
<td>Present</td>
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<tr>
<td>Round, or irregular 0.3 µm - 1 µm, Oval 0.8 µm - 1 µm, Rods 1 µm - 1.5 µm x 0.3 µm</td>
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<td>Rabbit</td>
<td>Yamagishi (1959)</td>
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<td>Tubules 0.2-0.4 μm wide</td>
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<td>Ito &amp; Shibasaki (1968)</td>
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<td>densities</td>
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<td>Human</td>
<td>Ito &amp; Shibasaki (1968)</td>
<td>Present</td>
</tr>
<tr>
<td>Rat</td>
<td>Rouiller et al. (1967)</td>
<td>Present</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Tanikawa et al. (1965)</td>
<td>Present</td>
</tr>
<tr>
<td>Mouse</td>
<td>Ito &amp; Shibasaki (1968)</td>
<td>Present</td>
</tr>
<tr>
<td>Human</td>
<td>Hampton (1958)</td>
<td>Present</td>
</tr>
<tr>
<td>Rat, Frog</td>
<td>Wood (1963)</td>
<td>Present</td>
</tr>
<tr>
<td>Rat, Calf</td>
<td>Wood (1963)</td>
<td>Present</td>
</tr>
<tr>
<td>Rat</td>
<td>Wisse (1970a)</td>
<td>Present</td>
</tr>
<tr>
<td>Human</td>
<td>Wisse (1970a)</td>
<td>Present</td>
</tr>
</tbody>
</table>
the ingestion of injected material by a previously undifferentiated lining cell.

Nathan (1908) and Fawcett (1955) do not recognise any structural features which justify dividing the cell population into more than one category of endothelial cell. However, division into phagocytic Kupffer cells and endothelial cells has been proposed by some authors. Zimmermann (1923, 1928) describes an endothelial sinusoidal wall with endocytic Kupffer cells sticking to it. Tanikawa et al. (1965) report that the Kupffer cells are larger, wider and contain more organelles than the endothelial cells. Yamagishi (1959) considers that the Kupffer cell is not involved in forming the wall of the sinusoid to which it is anchored and he draws attention to their numerous pseudopodia. Like Tanikawa et al., Yamagishi also noted the larger and more numerous organelles in the Kupffer cell of the rabbit; in particular, rough surfaced endoplasmic reticulum (RER) is present and the Golgi complex and mitochondria are larger than in adjacent endothelial cells. Schmidt (1960)
classifies the sinusoidal lining cells of mice into three types and his Type III corresponds to the Kupffer cell of other authors in that it is larger and has an oval nucleus with a nucleolus and irregularly distributed dense material in the nucleoplasm. The fullest description of these cells is given by Ito & Shibasaki (1968) who identify the Kupffer cells because they are large, rounded and bulge into the sinusoidal lumen; both surfaces possess numerous pseudopodia, but more are present on their luminal aspects.

The above distinctions are made on control specimens and many reports describe the changes which follow stimulation of phagocytic activity. Knisely, Bloch & Warner (1948), Altschul (1954) and Bloch (1955) can only distinguish various degrees of activity, but Zimmermann (1928) seems able to differentiate non-phagocytic endothelial cells from active Kupffer cells. Wisse (1970b) distinguishes between endothelial and Kupffer cells in two ways; endothelial cells have fenestrated extensions forming sieve plates
which are never seen in Kupffer cells. Also, after thorotrast administration, particles do not attach to endothelial cell surfaces, but do to Kupffer cells. Wisse observes that all the lining cells phagocytose, but Kupffer cells to a much greater extent than endothelial cells. 

As there are clear structural differences between these sinusoidal lining cells and endothelial cells in other tissues, they will be referred to in this study as lining cells. Those lining cells which are large and intensely phagocytic will be called Kupffer cells.

1.1.2 Space of Disse

This is a space of variable width between the lining and parenchymatous cells (Disse, 1890), sometimes extending between the latter as far as the junctional complex at a bile canaliculus. It contains reticulin fibres, unmyelinated nerve fibres enclosed in Schwann cells (Ito & Shibasaki, 1968) and occasionally a type of connective tissue or fat storing cell (Figure 1).

Reticulin fibres form an open-meshed
basket around the sinusoid. Each fibre is 30-70 nm in diameter, with the usual 64 nm periodicity, and they tend to be grouped into small bundles, embedded in amorphous material and situated between the microvilli extending from the parenchymatous cells.

Cells in the space of Disse, which are not lining cells or hepatic parenchymal cells, are also present. Small round cells, possibly lymphocytic or haemopoietic, are mentioned by Ito & Shibasaki (1968). Schnack, Stockinger & Wewalka (1966, 1967) note cells resembling fibroblasts in human liver, and these cells increase in conditions causing hepatic fibrosis. Others are impressed by the lipid content of these cells and they are described as fat-storing cells by Tanikawa et al. (1965) in the rat, Yamagishi (1959) in the rabbit and Ito & Nemoto (1952), Novikoff & Essner (1960), Stockinger (1966) and Ito & Shibasaki (1968) in the human. As these cells may have diverse functions, they will be called perisinusoidal cells, a term which only indicates their position.
Perisinusoidal cells are very variable in shape and can be in the clefts between parenchymatous cells, but are never in direct contact with the sinusoidal lumen (Ito & Shibasaki, 1968). Their large, oval nuclei may be indented by fat droplets (Yamagishi, 1959). Centrioles are not reported but a Golgi complex lies on one side of the nucleus. RER is present and may be a prominent constituent containing granular material as seen in normal fibroblasts (Yamagishi, 1959; Schnack et al., 1967). Large numbers of fat droplets, 0.8-2.9 μm in diameter, may be present in the perikaryon and arranged like a string of beads in the tenuous extensions of these cells. Although the membrane around each droplet is poorly defined, contiguous droplets do not fuse together (Ito & Shibasaki, 1968). The tortuous surface membrane carries microvillous projections and pinocytotic vacuoles; the surface is in contact with reticulin fibres which usually separate it from the parenchymatous cells and which may completely surround the perisinusoidal cells.
1.1.3 Vascular surface of hepatic parenchymal cells

Fawcett (1955) first describes the irregular, branching microvilli of variable length which project from the parenchymatous cells into the space of Disse. Ito & Shibasaki (1968) note that the number of microvilli decreases if the fibrous tissue content rises.

At the bases of the microvilli is the plasma membrane of the parenchymal cell.

1.2 Phagocytosis

The cells of the RES are distinguished by their extraordinary ability to engulf particles from the lymph and blood plasma. This forms the first line of defence against invading micro-organisms, as well as filtering from these fluids any circulating dead cells or foreign particles. Phagocytosis is the process by which particles are engulfed and is used as a general term including pinocytosis. Some workers consider that the only difference between these two processes is in the volume of fluid intake, with pinocytosis being a "drinking" process.
(Lewis, 1931; Wiener, Spiro & Margarettken, 1964), while others differentiate according to particle size (Karnovsky, Shafer, Cagan, Graham, Karnovsky, Glass & Saito, 1966). They are however, both active transport mechanisms for the intake of water, solutes and particles (Gosselin, 1967).

1.2.1 Clearance

Colloidal particles injected into the circulation are cleared within a few hours by RES. Radio-active colloidal iron and thorium dioxide in plasma drop by half within 6 hr after intravenous (IV) administration, with none left in 24 hr (Easton, 1955; Seno, Awai, Kobayashi, Ose & Kimoto, 1962). At the latter time, 60% of the dose is found in liver, 15% in spleen, 3% in lungs and 20% in skeleton (Easton, 1955; Kabisch, 1967). The proportion in liver rises with time, corresponding to a fall in the proportion in spleen. Easton (1955) believes that all macrophages may travel to the liver, and that Kupffer cells are normally produced in spleen, with lungs serving as an organ of excretion.
for macrophages moving out of the liver, at least when they are loaded with foreign matter.

The phagocytic properties of RES cells are shown, in rats, to develop progressively during foetal life (Reade & Jenkin, 1965). Cells preparing for division, and those recently divided, as shown by their uptake of tritiated thymidine, can phagocytose (Kelly, Brown & Dobson, 1962).

1.2.2 Uptake of particles

Particle uptake by a cell takes place in several stages; initial reversible adsorption of the particle to the cell surface, followed by irreversible engulfment and accumulation of the particles in vacuoles (Gordon & King, 1960; Gosselin, 1967; Wiener et al., 1964).

Fenn (1922) analyses the early stage of phagocytosis as a surface tension phenomenon. He postulates a fall in free surface energy of the cell, or the interface of the cell and particle, when a particle is ingested. Carr (1968) discusses whether adhesion of particles to the macrophage cell coat is due to some carbohydrate-rich layer such as that
found in many other cells. Particles are seen aligned along a membrane and Bennett (1956) in his membrane flow theory discusses the concept of non-specific, or specific, binding sites on the cell membrane, as well as ionic attraction. He envisages a cleft forming in the membrane, with the source at the cell surface and a sink at the bottom of the cleft. The membrane forms at the source and flows to the sink where it is destroyed, and so any particle attaching to it near the source will be drawn toward the sink and so enter the cell.

The engulfment of large particles, e.g. bacteria, or 1 μm aggregates of smaller particles is described by Casley-Smith & Read (1965). After adsorption to the cell surface, pseudopodia are extended on either side of the particle and eventually meet, to engulf it in a vacuole. This is named "active en-membranosis" by Tanaka (1961) who also describes "passive en-membranosis" similar to vesiculation of Bennett (1956). In this process 5-50 nm particles lie near the cell membrane which indents,
drawing in and engulfing the particles. This gives rise to large numbers of small vesicles which coalesce and so form vesicles of 1 μm diameter (Casley-Smith & Reade, 1965). They note the appearance of these more frequently in endothelial cells, particularly Kupffer cells, than parenchymal cells.

The appearance of worm-like bodies in Kupffer cells has led to the concept of micropinocytosis vermiformis (Törö, Ruzsa & Rohlich, 1962; Orci, Pictet & Rouiller, 1967; Rouiller, Pictet, Nicolescu & Orci, 1967; Matter, Forsmann & Rouiller, 1968). These are seen after dosing rats with Indian ink, tetracycline and streptozotocine, or after partial hepatectomy, while Rouiller et al. (1967) also report seeing them in control rats.

The diameter of the worm-like bodies varies from 0.1-0.15 μm and they are either connected to the vascular surface of the cell or, as described by Matter et al. (1968), associated with pinocytotic sacs. The external coat is an osmiophilic membrane similar to the cell membrane, and its lumen is filled with
moderately dense cross-striated material. A darker central line does not extend to the rounded ends. Circular profiles occur, with dense cores and occasionally coated vesicles are budded from the outer membrane; these may be transporting material across the membrane into the cytoplasm. No connection with ER is seen. Their constant diameter is thought to be due to 0.5 μm thick external coats duplicated by folding and the cross-striations to be due to proteinous rods lying perpendicular to the surface (Törö et al., 1962). The central density may be due to osmiophilic groups at the ends of the rods to which particles stick before phagocytosis. This line is obscured after ingestion of carbon. Matter et al. (1968) establish, from serial sections, that micropinocytosis vermiciformis consists of an intricate system of ramifying tubular and lamellar cavities; that all parts are in communication with extracellular space and that the whole system is surrounded by, and in communication with, coated vesicles. They suggest that this system may signify intensive
pinocytosis and be a feature of all RES cells.

1.2.3 Factors affecting uptake

In liver, the most actively phagocytic cells are the Kupffer and endothelial cells, and the activity of these cells is shown to be affected, sometimes markedly, by various circulating substances.

Hormones are known to have a powerful effect on RES activity (Kelly, Dobson, Finney & Hirsch, 1960; Nicol, Vernon-Roberts & Quantock, 1965, 1966; Nicol & Vernon-Roberts, 1965; de Mignard, Patek & Bernick, 1966); oestrogen, oestrone, oestriol and 17-β oestradiol are all powerful RES stimulants, while progesterone is a mild stimulant, cortisone stimulates in small doses, anterior pituitary hormones stimulate, thyroxine potentiates the effect of oestrogen and posterior pituitary hormones have no effect (Nicol et al., 1965).

Heller, Meier, Zucker & Mast (1957) suggest these effects are brought about by the hormones altering surface membrane phenomena. Other RES stimulants are zymosan - a yeast cell wall product - and bacterial endotoxin (Kelly et al.,
1960; Nicolescu & Rouiller, 1967). Kelly et al. (1960) suggest the enhanced uptake of particles is due to proliferation of the cells as shown by increased DNA uptake of liver in such conditions. On the other hand, Heller et al. (1957) attribute the increase in uptake to enhanced efficiency of pre-existing cells. It is reported that oral and colloidal iron is taken up more efficiently by the liver if it is preceeded by oral N-2 Fluorenlyacetamide (Kent, Minick, Volini, Orfei & de la Huerga, 1963).

Stuart (1970) reports the rate of particulate material removal is enhanced following glycerol trioleate, and ethyl oleate caused a depression of this activity. Lowering of phagocytic activity is brought about by oral or parenteral administration of lipids such as methyl palmitate, trioleate or ethyl stearate. It is unclear how this effect is mediated (Di Luzio & Blickens, 1966; Berken & Benacerraf, 1968). Thymectomy and whole body irradiation are reported not to alter phagocytic activity by Fridrich & Schafer (1966).

Surface active agents are employed by many
workers to study the activity of RES cells (Berry, Starr & Haller, 1949; Asiddao, Filkins & Smith, 1964; Karnovsky, Graham, Karnovsky, Saito, Shafer & Glass, 1967). A marked rise in phagocytosis follows additions of agents such as sodium glycocholate ($5 \times 10^{-3}$%) and taurocholate ($5 \times 10^{-2}$%), Tween 80 ($5 \times 10^{-4}$%) and Triton X-100 ($5 \times 10^{-3}$%).

Particles and cells are affected to different degrees by surface active agents (Berry et al., 1949). From this hypothesis, Asiddao et al. (1964) interpret the change brought about by the surface active agents detailed above as meaning the major effect is shown on the cells rather than on the particles, since lowering surface tension increases phagocytosis. When stronger concentrations of Tween ($5 \times 10^{-2}$%) are used phagocytosis is depressed, and the effect is showing predominantly on the particles.

Engulfment of particles by cells is shown to be an energy dependent process; some energy is supplied in Kupffer cells, as in polymorphonuclear leucocytes and monocytes, by glycolysis, which is demonstrated by
iodoacetate inhibition of glycolysis being associated with a reduction in phagocytosis (Sbarra & Karnovsky, 1959; Asiddao et al., 1964; Karnovsky et al., 1966). Phagocytosis is reported to be equally efficient under aerobic or anaerobic conditions (Sbarra & Karnovsky, 1959). On the other hand, Yokomura, Seno, Sogabe, Nakatsuka & Kubo (1967) suggest the depression may only be due to the swelling of the cell when such inhibitor is introduced. There is a reduction in phagocytosis also following inhibition of oxidative phosphorylation by 2-4 DNP, suggesting a similarity to alveolar macrophages (Oren, Farnham, Saito, Milofsky & Karnovsky, 1963; Asiddao et al., 1964). Hirsch (1965a) reports an increased turnover of neutral lipids and phospholipids as shown by uptake of acetate and $^{32}$P which Karnovsky (1962) thinks may reflect breaking and making of membrane links. Inhibition by uranyl nitrate is reported by Gordon & King (1960) who suggest two mechanisms of action; the ion may combine with the protein portion of cell membrane rendering it impermeable, and interfere with
the reversible unfolding processes of phagocytosis, or it may affect respiration and thus carbohydrate metabolism.

So, uptake appears to be a surface tension phenomenon, with surface active agents producing marked effects on it. The actual passage of the particle into the cell is interpreted as being mediated either by membrane flow, pseudopodial engulfment or micropinocytosis vermiformis, all of which appear to be energy dependent processes. After engulfment, particles are seen in vacuoles of varying shapes and sizes known as phagosomes or phagocytic vacuoles, which become the target for lysosomal activity.

1.2.4 Blockade

If the RES is presented with a large quantity of colloidal material it can phagocytose it, but another similar load within a short time is not engulfed so efficiently. However, if a different type of particle is presented to it, its uptake is enhanced. This is known as blockade of the RES and has led to the concept of opsonin involvement,
opsonins being proteins of antibody or complement nature in the serum (Berry & Spies, 1949; Hirsch, 1965a; Rowley, 1966a, b; Wisse, 1970b).

Jenkin & Rowley (1961) and Jeunet & Good (1967) report the restoration of phagocytic activity to RES cells after blockade by addition of plasma, and Pisano, Filkins & Di Luzio (1968) find isolated Kupffer cells require serum before they will phagocytose. This evidence implicates plasma factors in phagocytic activity; these are highly specific and capable of distinguishing between different types of particle in the blood (Saba & Di Luzio, 1966; Jeunet, Cain & Good, 1968). Megirian, Kitchen, Laffin & Leonardi (1968) suggest that the opsonin activity is in the gamma globulin fraction of serum. Saba & Di Luzio (1966) report that the opsonin system has more importance in the uptake of large particles than small and suggest the role of these proteins is in facilitating cellular recognition of the particles before ingestion.

1.2.5 Distribution of phagocytosed material

Different groups of workers give
conflicting reports regarding the distribution of phagocytosed material. Details are given in Table 2. In young rats, Frankel, Patek & Bernick (1962) report distribution of carbon in Kupffer cells throughout the liver lobules within 1 d. After 21 d giant multinucleated carbon-containing cells are seen. Bernick, Patek & de Mignard (1966), on the other hand after 30 d, report aggregations in giant cells only in the periphery of lobules.

In 1955, Easton uses thorium dioxide in adult mice and reports Kupffer cells around central veins loaded within 5 d. De Mignard, Patek & Bernick (1966) inject adult rats with carbon and after 3 m find loaded macrophages in periphery of lobules. After 1 m Patek, de Mignard & Bernick (1967) have a similar finding of carbon in giant cells in the outer third of the lobules.

1.3 **Lysosomes**

Primary lysosomes are widely distributed vesicles containing enzymes which can digest particles normally engulfed by the cells.
<table>
<thead>
<tr>
<th>Colloid</th>
<th>Animal</th>
<th>Age</th>
<th>Time After Colloid Treatment</th>
<th>Distribution</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thorium dioxide</td>
<td>Mouse</td>
<td>Adult</td>
<td>5 d</td>
<td>Kupffer cell around central vein loaded.</td>
<td>Easton (1955)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>11 d</td>
<td>Loaded Kupffer cells evenly distributed through lobes.</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td></td>
<td>1 d</td>
<td>Kupffer cells - some loaded, others with 1 or 2 aggregations.</td>
<td>Driessens, Demaille &amp; Rohart (1967)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 wk</td>
<td>15 min</td>
<td>Some seen in liver firstly in peripheral rim of vacuoles, then these become homogeneous</td>
<td>Grampa (1967)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3 d</td>
<td>Larger, denser aggregations, with confluence of vesicles, obscuring</td>
</tr>
<tr>
<td>Colloid</td>
<td>Animal</td>
<td>Age</td>
<td>Time After Colloid Treatment</td>
<td>Distribution</td>
<td>Authors</td>
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<td>------------------------------------------------------------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Thorium dioxide</td>
<td>Rat</td>
<td>8 wk</td>
<td>3 d</td>
<td>cellular detail. Kupffer cells show more than parenchymal.</td>
<td>Carr (1968)</td>
</tr>
<tr>
<td>Thorotrast</td>
<td>Mouse</td>
<td></td>
<td>1 hr</td>
<td>Kupffer cells contain thorotrast in vacuoles and dense bodies.</td>
<td>Kluge &amp; Hovig</td>
</tr>
<tr>
<td>&quot;</td>
<td>Rat</td>
<td>Adult</td>
<td>6 and 12 hr</td>
<td>Most Kupffer cells show some particles. Vacuoles around periphery of parenchymal cell show some</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>24 hr</td>
<td>In Kupffer cell vacuoles and thereafter accumulated in lysosomes often with mitochondria in close proximity. In various dense bodies in parenchymal cells.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 d</td>
<td>Particle concentration in parenchymal cells falls. Some in peribiliary dense bodies.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7 d</td>
<td>None seen except some in bile lumen.</td>
<td></td>
</tr>
<tr>
<td>Colloid</td>
<td>Animal</td>
<td>Age</td>
<td>Time After Colloid Treatment</td>
<td>Distribution</td>
<td>Authors</td>
</tr>
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<td>--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>Carbon</td>
<td>Rat</td>
<td>Young</td>
<td>1 d</td>
<td>In Kupffer cells throughout lobules. C-laden macrophages in central and hepatic veins occasionally.</td>
<td>Frankel, Patek &amp; Bernick (1962)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 d</td>
<td>More C in lobules. Kupffer cells more heavily laden.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>21 d</td>
<td>Fall in individual C-containing macrophages in sinusoid. Rise in multinucleated giant cells containing much C.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5 m</td>
<td>C in various aggregations in parenchymal cells, sometimes near bile canaliculus.</td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td>Rat</td>
<td>8 wk</td>
<td>30 d</td>
<td>Aggregations of C in giant cells in periphery of lobules.</td>
<td>Bernick, Patek &amp; de Mignard (1966)</td>
</tr>
<tr>
<td>&quot;</td>
<td></td>
<td></td>
<td>104 wk</td>
<td>Medium or larger non-cellular C aggregates scattered through</td>
<td></td>
</tr>
<tr>
<td>Colloid</td>
<td>Animal</td>
<td>Age</td>
<td>Time After Colloid Treatment</td>
<td>Distribution</td>
<td>Authors</td>
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<tr>
<td>---------</td>
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<td>-------------------------------</td>
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<td>------------------------------</td>
</tr>
<tr>
<td>Carbon</td>
<td>Rat</td>
<td>104 wk</td>
<td>30 d</td>
<td>lobules. Fewer Kupffer cells with particles than in young liver.</td>
<td>de Mignard, Patek &amp; Bernick (1966)</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td></td>
<td>3 m</td>
<td>C-loaded macrophages in periphery of lobules. Noncellular carbon masses.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>5 m</td>
<td>1 m</td>
<td>C masses in areas adjacent to hepatic and portal veins, in adventitia of vessels and encapsulated by connective tissue. C-loaded macrophages in sinusoids.</td>
<td>Patek, de Mignard &amp; Bernick (1967)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24 m</td>
<td>1 m</td>
<td>Peripheral ½ of lobes show large aggregates of C in giant cells in portal triad and sinusoids. Central zone shows uniform distribution of C-laden macrophages.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Less individual C-laden macrophages in</td>
<td></td>
</tr>
<tr>
<td>Colloid</td>
<td>Animal</td>
<td>Age</td>
<td>Time After Colloid Treatment</td>
<td>Distribution</td>
<td>Authors</td>
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</tr>
<tr>
<td>Carbon</td>
<td>Rat</td>
<td>24 m</td>
<td>1 m</td>
<td>sinusoids. More giant cells containing much C. Many sinusoidal cells C-free and desquamated. C in endothelia of central and hepatic veins. Free C and C-laden macrophages in adventitia and lumina of hepatic collecting veins, but not in portal system.</td>
<td>Casley-Smith &amp; Reade (1965)</td>
</tr>
<tr>
<td>Carbon and thorium</td>
<td>Rat</td>
<td>Adult</td>
<td>1 min</td>
<td>In sinusoid lumen. &quot;endothelial cells. Aggregations in large vesicles.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5 min</td>
<td>Bacteria showing signs of digestion.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15 min</td>
<td>Disruption of some bacteria.</td>
<td></td>
</tr>
<tr>
<td>Bacteria</td>
<td></td>
<td></td>
<td>25 min</td>
<td>Kupffer cells contain ink.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>125 min</td>
<td>Lead aggregations in Kupffer and parenchymal cells very variable.</td>
<td></td>
</tr>
<tr>
<td>Indian Ink</td>
<td>Mouse</td>
<td></td>
<td>5 min</td>
<td></td>
<td>Onoe &amp; Tsukada (1964)</td>
</tr>
<tr>
<td>Lead</td>
<td>Rabbit, Mouse</td>
<td></td>
<td>7 d</td>
<td></td>
<td>Causey (1965)</td>
</tr>
</tbody>
</table>
Organelles and blood cells which have become obsolete are also digested by lysosomes, and in some pathological conditions lysosomal enzymes may be released into the cell and destroy it.

De Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955) give the original description of lysosomes. They are spherical, 0.25 μm in diameter (Allison, 1967) and observed to have a single membrane surrounding them (Confer & Stenger, 1964; Novikoff, Essner & Quintana, 1964; de Duve & Wattiaux, 1966; Arstila & Trump, 1968; Daems, Wisse & Brederoo, 1969).

After a cell has ingested particles into a phagosome, it fuses with a primary lysosome, thus forming a secondary lysosome in which digestion of the contents takes place. The static morphological and dynamic biochemical evidence supporting this concept is reviewed by Ericsson, Trump & Weibel (1965), Straus (1967) and Ericsson (1968). Those substances which can be utilised diffuse out into the cytoplasm; during starvation autolysis of liver can return amino acids to the body pool for
essential protein synthesis at other sites (Swift & Hruban, 1964). During this digestion process, secondary lysosomes move about the cytoplasm, becoming smaller and denser probably through dehydration, and may fuse with other primary lysosomes replenishing the enzymes. Lysosomal contents which cannot be metabolised are retained within the membrane forming a residual body (de Duve, 1963, 1967; de Duve & Wattiaux, 1966; Allison, 1967; Dingle, 1968). This may be excreted by parenchymal cells into a bile canaliculus by a process of reverse phagocytosis or defaecation (Hampton, 1958; de Duve, 1964; 1967, 1969; Bradford, Elchlepp, Trump & Kinney, 1968; Dingle, 1968; Kluge & Hovig, 1969).

Intracellular digestion is taking place continuously, but it increases in some pathological processes when lysosomal membranes rupture and release their enzymes into the cytoplasm (Weissman, 1964, 1965; Allison, 1967). This can result in inflammation, necrosis or allergies (Hirsch, 1965b). Factors known to render lysosomes more fragile are Vitamin A, ultraviolet light and endotoxin
(Lucy, Dingle & Fell, 1961) while cortisone stabilises their membranes (Weissman, 1964).

1.3.1 Enzymic contents of primary lysosomes

The contents of primary lysosomes include acid phosphatase, acid RNAse, acid DNA-ase, acid protease, phospholipidase, mucopolysaccharides and glycoprotein, plasminogen activator, protease, haemolysine and unidentified basic protein (Pariente, 1966). Allison (1967) also reports the presence of a pyrogen and Aronson & Davidson (1965) find hyaluronidase. The optimum pH of the acid hydrolases is pH 5 (Gahan, 1967; Coffey & de Duve, 1963).

The enzymes are stored in granular form (Hirsch & Cohn, 1964; Zucker-Franklin & Hirsch, 1964; Hirsch, 1965a, b; Cattan, 1966; Allison, 1967).

Lysosomes are widely distributed throughout the tissues; in liver they can be shown by acid phosphatase staining in parenchymal and endothelial cells (Gomori, 1941; Goldfischer, Essner & Novikoff, 1964; Sabatini, Miller & Barrnett, 1964; Janigan, 1965; Fahimi & Drochmans, 1968). In mouse liver, De Man, Daems, Willighagen & Van Rijssel (1960) report
that most lysosomes lie between the parenchymal cell nucleus and the bile canaliculus, and in Kupffer cells. Straus (1963) reports a study in which he administered horse-radish peroxidase and stained for acid phosphatase and found acid phosphatase (red) in the peribiliary region. Staining for peroxidase showed phagosomes (blue) in the sinusoidal side of the cell. He gives evidence that the two types of vacuoles fuse more readily in Kupffer cells, because the purple colouration which he takes to indicate fusion is most abundant in Kupffer cells. This could also be interpreted as the close proximity of many small red and blue deposits in small cells making them appear mixed.

Increased phagocytic activity is reported to give rise to increased acid phosphatase activity (Golberg, Martin & Smith, 1960; Meijer & Willighagen, 1961). After intraperitoneal administration of glucagon, there is evidence of an increase in lysosomal number (Ashford & Porter, 1962; Arstila & Trump, 1968).
1.3.2 **Origin of lysosomal enzymes**

There is still conjecture about the site of lysosomal enzyme synthesis, but it is suggested that they are synthesised in ER (Novikoff, 1963; Novikoff, Essner & Quintana, 1964; de Duve & Wattiaux, 1966; Cohn, Fedorko & Hirsch, 1966; Allison, 1967; Dingle, 1968; Arstila & Trump, 1968; Cohn & Fedorko, 1969).

They may be formed in a similar way to protein secretion granules and are first found in the cisternae of RER after possible synthesis on the attached ribosomes. Smooth surfaced extensions of ER could be used for their transport to the Golgi area and there is evidence that this process is energy dependent. Arstila & Trump (1968) gave ethionine to cells, which lowers ATP in the cell, and found that even after glucagon administration, formation of autophagic vacuoles was inhibited. This they interpret as ATP being required either for enzyme transport from ER to Golgi area, or for wrapping of ER around organelles to form autophagic vacuoles. They further believe that the Golgi area is the source of hydrolases, because this area shows
acid phosphatase activity, new autophagic vacuoles are seen in that region, and they see suggestions of fusion between Golgi vesicles and double walled autophagic vacuoles. Novikoff (1963), Novikoff, Essner & Quintana (1964), Cohn, Fedorko & Hirsch (1966) and Bartók, Tótović & Gedigk (1967) postulate that in the Golgi area enzymes are packaged into small vesicles which represent the primary lysosome.

Those areas of ER supplying the Golgi area with lysosomal enzymes may directly form lysosomes in the form of dense bodies and of autophagic vacuoles (Novikoff, Essner & Quintana, 1964). It is not known how enzyme formation is controlled, but synthesis is related to phagocytosis which leads de Duve & Wattiaux (1966) to speculate that phagocytic vacuoles in the centrosome region may cause discharge of lysosomes in that region which in turn may trigger the synthesis of more hydrolases.

1.3.3 Origin of membranes

Membranes in a cell can be classified as thin (6-7 nm) or thick (9-10 nm); thin
membranes are seen in RER and SER, in forming
Golgi cisternae, mitochondrial outer and inner
membranes and inner and outer nuclear
membranes and microbodies. The thick membranes are in
secondary lysosomes, maturing Golgi cisternae,
vesicles and vacuoles and plasma membranes.
In inner limiting membranes of double walled
autophagic vacuoles, three size classes are
found; first thin, second thicker than the
plasmalemma class and third intermediate
(Arstila & Trump, 1968).

There is much speculation about the
origin of the membrane of autophagic vacuoles.
De novo synthesis is suggested by Ashford &
Porter (1962), but the majority of authors
think that preformed membranes are utilised
(Novikoff et al., 1964; Ericsson, 1965;
Thinès-Sempoux (1967) draws attention to the
similarity of chemical composition between
lysosomal and plasma membranes. Histochemical
data suggests an origin either in ER or some
Golgi lamellae since all these lack
thiamine pyrophosphate activity (Arstila & Trump, 1968). In liver, autophagic vacuoles are smooth, so SER or Golgi membranes could be the source of membranes. Since Golgi cisternae often contain liposomes, and SER and autophagic vacuoles do not, SER is regarded as the most likely source of autophagic vacuole membranes.

Double membranes are seen around some autophagic vacuoles, sometimes both thin, or inner may be thick. All autophagic vacuoles with thick membranes show acid hydrolase activity, whereas those with thin membrane do not, so the thin-thick membrane is regarded as a later stage (Arstila & Trump, 1968).

Under certain circumstances Golgi vesicles, or primary lysosomes, fuse with plasma membrane or membrane of phagocytic vacuoles (Dingle, 1968). The plasma membrane may be modified by opsonins to allow this fusion, but not fusion with other organelles. Dingle postulates that the Golgi vesicles have high surface tension, facilitating fusion and that opsonins which diminish membrane stability also produce a suitable acceptor
surface for fusion with Golgi vesicles.

Degranulation of primary lysosomes following phagocytosis is reported (Cohn, 1963; Lockwood & Allison, 1963; Zucker-Franklin, 1964; Hirsch, 1965a). The enzymes are discharged following fusion with a phagosome and the contents of the phagosome are digested by the enzyme and removed for use elsewhere.

1.4 Iron metabolism

An adult human male has total body iron of about 4 g. Of this, 3 g is accounted for in Hb, myoglobin and iron-containing enzymes, the other 1 g in the non-haem storage forms of iron, ferritin and haemosiderin. Normally, iron is available in the diet, the average daily intake being about 10-15 mg, of which 10% is absorbed, so 1 mg is absorbed daily (Fielding, 1967). Blood loss and, therefore, iron loss, is normally low in males, and iron is not excreted to any great extent, so a positive iron balance may be maintained provided a normal diet is available.

There are greatly increased iron demands in females due to menstruation, pregnancy and
lactation, with their daily requirement being about twice that of males. If insufficient iron is made available from the diet, iron stores are mobilised, and if this is allowed to continue, iron deficiency anaemia will result. If iron stores become depleted, spontaneous recovery from haemorrhage cannot occur, nor can physiological polycythaemia, for example in pulmonary insufficiency. So it is obvious that iron is a small, but vitally important constituent of the diet.

1.4.1 Iron absorption

Iron in its ferrous form is absorbed most readily through the mucosa of duodenum and upper small bowel (Bothwell & Finch, 1962). The transfer across mucosal cells is known to involve oxidative metabolism, but details of this remain unknown. Entry of iron into a mucosal cell stimulates production of the protein apoferritin which combines with the iron to form ferritin. These mucosal cells can act as a temporary store for iron as ferritin, but usually the iron is transferred rapidly to the plasma where it is carried by
the protein, transferrin, to its destination. The amount of iron absorbed from the intestine depends on both the size of the iron store in the body and the rate of haemopoiesis (Bothwell & Finch, 1962; Walsh, Cantrill & Sanford, 1963). Callendar (1964) shows mean absorption of inorganic iron is 30% in non-anaemic and 56% in iron deficient subjects, and absorption of haemoglobin iron is respectively 13% and 25%. In all cases a dose of 5 mg iron, or equivalent of haemoglobin, was given. The controlling mechanism is obscure. It is also dependent on the other contents of the diet and Sorensen (1964) shows that if protein is included with the iron less is absorbed than if it had been on its own or with glucose. The effects of digestive juices on iron absorption have been studied. Goldberg, Lochhead & Dagg (1963) show that absorption may be depressed by achlorhydria. Davis & Badenoch (1962) show evidence in humans that pancreatic secretion contains some substance which inhibits iron absorption. Ascorbic acid is shown to increase iron absorption (Moore, Bierman,
Minnich & Arrowsmith, 1940), and sodium phytate to lower absorption of inorganic iron, although it does not affect absorption of haemoglobin iron (Turnbull, Cleaton & Finch, 1962).

Transferrin is a glycoprotein of M.W. 83,000, with each molecule containing two iron atoms in the ferric state (Bearn & Parker, 1964). Fifteen different types of transferrin have been identified, normal transferrin being designated transferrin C, that migrating faster, B, and that slower, transferrin D. Transferrin comprises 3% of total plasma protein (Frankel, 1961) and experiments done by Wheby & Jones (1963) indicate that the level of transferrin saturation has no effect on the regulation of absorption. It is suggested that the function of transferrin is to carry the iron, then affix to the red blood cell precursors so that the iron can chelate onto cell membrane acceptors (Jandl, Inman, Simmons & Allen, 1959). Normally, iron found in plasma is coupled to transferrin. Abnormal iron complexes may be found in some conditions; severe intravascular haemolysis
results in haemoglobin in plasma, and liver cell necrosis can result in ferritin in the circulation and iron in the ferric form occurs in plasma after acute iron poisoning. The only other occasion when iron is found in the plasma is after parenteral iron therapy when iron dextran or saccharate complexes are found, and these complexes are rapidly cleared by the RES.

1.4.2 Kinetics

As well as dietary intake of iron, the RES provides a proportion of the iron requirement by salvaging iron from effete erythrocytes, and in the case of parenteral iron therapy it provides iron phagocytosed from the blood. Noyes, Bothwell & Finch (1960) track the route of radio-active erythrocytes; they report phagocytosis by RES cells, then iron is split from haemoglobin, part kept in the RES cell and the rest returned, via transferrin, for distribution throughout the body. In normal circumstances, this goes to the bone marrow. If iron therapy raises the plasma iron level, they notice no effect on the total amount of
iron released from the RES. This control system is unknown.

Pollycove (1964) shows the following system after doing radio-iron studies in the normal subject. Measurements of $\gamma$-ray emission were taken over the sacrum (marrow) and liver (iron stores).

MARROW

- Marrow labile pool: 85 mg
- Haem: 30 mg

STORAGE

- 1000 mg

ERYTHROCYTES

- 2700 mg

PLASMA

- 4 mg
Release of iron from RES is studied by Barry, Tallarida & Rusy (1968). Labelled haemoglobin in erythrocytes is recovered for re-use within 30 min of removal from the circulation. Seno, Awai, Kobayashi, Ose & Kimoto (1962) show the partial uptake from the blood of intravenously administered colloidal iron within 6 hr and total within 24 hr. Infused radio-active iron shows a delay of 2 wk before appearing in erythrocytes, and is found during erythrocyte sampling over several months, suggesting detention in RES; either the RES has a limited ability to deal with iron presented to it, or it may release it into a pool (Barry et al., 1968).

There is no significant excretion of iron and the slight loss that does occur is normally constant, due to the loss of epithelial cells. Spasmodic losses occur due to bleeding.

1.4.3 Ferritin

The storage forms of iron are ferritin and haemosiderin which make up about 25% of the body's iron (Bothwell & Finch, 1962) and 67% of the rest of the iron is in the form of Hb.
(Frankel, 1961). Shoden & Sturgeon (1958) report one of their studies on iron in which they give 2000 mg colloidal iron to rabbits over 6 wk and show the pattern of distribution of the two storage forms of iron over a 26 wk period. In that time they show a movement of iron from spleen and bone marrow to liver; the increase in the liver shows mainly in haemosiderin and there is a concurrent decrease in liver ferritin. Shoden & Sturgeon (1962) outline the following scheme for iron storage in rabbit livers:

\[
\text{Iron dextran} \rightarrow \text{Ferritin} \rightarrow \text{Insoluble iron} \\
\hspace{1cm}\text{granules} \\
\hspace{2.5cm}(\text{haemosiderin P}) \\
\hspace{2.5cm}\text{in parenchymal cell} \\
\hspace{1cm} \uparrow \hspace{1cm} \downarrow \\
\hspace{1cm} \text{Saccharated iron oxide} \rightarrow \text{insoluble iron} \\
\hspace{2.5cm}(\text{haemosiderin K}) \\
\hspace{2.5cm}\text{in Kupffer cell}
\]

Another study on liver iron after iron dosage is reported by Beaufay, Bendall, Baudhuin & de Duve (1959) in which they find all fractions of liver iron rise, the greatest rise being in the fraction containing nuclei.
and heavy mitochondria, showing that the iron is in the form of gross haemosiderin granules.

Ferritin and haemosiderin are very similar structurally and functionally; ferritin is diffusely scattered soluble iron, which can be crystallised, and haemosiderin is in insoluble aggregates and cannot be crystallised (Bothwell & Finch, 1962). Ferritin consists of a variable quantity of iron in a core surrounded by a protein shell of apoferritin whose MW is 460,000. Plasma iron is initially incorporated into ferritin with a low iron content; ATP and ascorbic acid mediate the transfer and there is formation of a complex involving 2 moles ATP, 1 mole ascorbic acid and iron-transferrin molecule (Mazur, Green & Carleton, 1960).

There is evidence that ferritin from different organs shows variations, designated isoferritins. Alfrey, Lynch & Whiteley (1967) describe tests on ferritin from spleen, liver, reticulocytes and bone marrow and find two ferritins in marrow, one with faster electrophoretic mobility than the other and
with different mobilities to those from the other three organs. The slower ferritin from marrow appears similar to that from spleen, and faster than that from reticulocytes. The mobilities for these ferritins from liver and spleen are identical with those of the corresponding apoferritins, which indicates that the differences are in the protein coat, and not in the iron content.

Iron injections can accelerate liver ferritin synthesis by a factor of 4-5 times (Fineberg & Greenberg, 1955a) or even 5-10 times (Yoshino, Schachter & Manis, 1966), without affecting the synthesis of other proteins (Saddi & Von der Decken, 1964; 1965). Fineberg & Greenberg show there is a rise in the protein moiety of ferritin and apoferritin and not merely a conversion of pre-existing apoferritin to ferritin. They estimate the turnover time of ferritin to be at least 80 hr. The same workers, Fineberg & Greenberg (1955b), show that the iron is not attached to the apoferritin until its synthesis is complete; so there is stimulation of apoferritin synthesis, with the
iron-free protein appearing, then the attachment of iron. They postulate that iron may have a direct action on the synthesis of apoferritin, possibly in a catalytic role. It could be that the ferric hydroxide of ferritin acts as part of a template in the apoferritin synthesising apparatus, influencing the conjugation of peptide chains forming the iron binding sites of the molecule. The other effect iron may have on apoferritin synthesis may be that in combining with the protein to form ferritin, apoferritin concentration falls and if its concentration inhibits more apoferritin forming, the inhibition is thus removed.

Biochemical evidence concerning liver ferritin synthesis following iron administration is confusing. Drysdale & Munro (1965a) interpret the increase in ferritin under such circumstances as being due to stabilisation of preformed ferritin, whereas Fineberg & Greenberg (1955a) show an increase in net synthesis of apoferritin.

There is also conflict in results reported by workers using actinomycin-D, which prevents
m-RNA synthesis. Drysdale & Munro (1965b) and Saddi & Von der Decken (1965) find no effect on the increase in apoferritin synthesis previously found in iron-treated animals; in 1966 Drysdale & Munro further report this effect, provided the actinomycin-D is used at a level sufficient to block only m-RNA synthesis, but not blocking general protein synthesis. Yu & Fineberg (1965) report 85% inhibition of leucine incorporation into ferritin in liver slices after actinomycin and a fall of 44-91% in apoferritin synthesis is reported by Yoshino, Schachter & Manis (1966). Thus gene-dependent synthesis of RNA is a pre-requisite for enhanced apoferritin synthesis in response to iron. Yoshino, Manis & Schachter (1968) did work to elucidate if iron acts via gene activation and synthesis of m-RNA or if its role is at subsequent stages of protein biosynthesis, i.e. translation of m-RNA, or stabilisation of protein intermediates. Yoshino et al. (1968) show by three lines of evidence that gene activation is involved in regulation of ferritin synthesis. Firstly, in vivo synthesis of ferritin was estimated and the
increase noted after iron administration was inhibited by actinomycin-D. Iron administration was shown to stimulate production of liver nuclear RNA as measured by uptake of $^{14}$C orotic acid, and addition of iron in vitro to liver slices, which would be expected to increase the rate of ferritin synthesis if iron influenced translation of m-RNA, failed to stimulate synthesis although it was taken up into the slices. Even though much work has been done on apoferritin synthesis, its controlling mechanism remains obscure.

The structure of the iron core of ferritin has been studied by many workers (Farrant, 1954; Richter, 1959; Muir, 1960; Frankel, 1961; Harrison, 1963; Wohler, 1964; Haggis, 1965; Harrison, Fischbach, Hoy & Haggis, 1967; Pape, Multani, Stitt & Saltman, 1968a,b; and Fischbach, Harrison & Hoy, 1969). Richter (1959) and Muir & Golberg (1961) describe cellular transformation of iron compounds into ferritin. Richter finds that within 4 hr of intraperitoneal injection of iron small numbers of ferritin molecules are seen alongside injected colloidal ferric hydroxide in sinus endothelial
cells of spleen and liver, splenic macrophages and some vascular endothelial cells in various renal capillaries. After 6 d there is much ferritin close to deposits of injected iron, surrounded by a simple membrane. Depending on the orientation of molecules in the electron beam, profiles of 2-4 particles of iron of about 3 nm diameter are seen. Richter calculates that each of these, when the iron content of ferritin is 23%, contains 600 atoms of iron, possibly in chains of FeOOH forming micelles. Muir (1960) also shows different profiles of the ferritin molecule and calculates 300 atoms per micelle. Richter (1959) shows the ferritin molecule is 9.4 nm in diameter and Harrison et al. (1967) describe the iron core as 7.5 nm and a protein coat of 2.5 nm. The iron is (FeOOH)$_8$ ($\text{Fe}_5\text{O}_7\text{P}_3\text{H}_2$). Harrison et al. (1967) state that \textit{in vivo} evidence suggests that apoferritin is formed first in biosynthesis and this supports Fineberg & Greenberg's (1955a) observation on \textit{de novo} synthesis of apoferritin following administration of ferrous salts. The iron, in
the ferrous state then enters the protein shell where it becomes oxidised to ferric and precipitates inside the shell as ferric oxyhydroxide. These crystallites are either bound to the protein or are too large to move out through the subunits which do allow the passage of ferrous iron. The diffraction pattern of ferritin iron, although not identical to any known mineral bears a strong resemblance to that of ferric oxyhydroxide. This molecule consists of close packed layers of oxygen atoms, with iron atoms in interstitial holes.

In 1969, Fischbach et al. conclude that the shape of the mineral in full ferritin molecules is largely imposed by the shape of the interior of the protein, while the atomic structures of core and protein are not specifically related. They think that the iron may grow within different protein molecules at a number of different sites in a number of directions.

The opposite view, that the mineral is formed before the protein in ferritin, is held by Pape et al. (1968a,b). Fischbach et al. (1969)
feel this is unlikely, since it would mean the atomic structure of the iron would be expected to bear a specific relationship to its external shape.

Harrison (1963) proposes an approximately spherical shell of 20 protein subunits, each of MW 24,000 measuring 7.4 nm diameter, confirmed by Fischbach & Anderegg (1965). Within the protein Haggis (1965) suggests the iron is present in the form of small crystallites, even as a single small crystal in some molecules and he calculates that if 0.035-0.042 nm$^3$ per FeOOH is allowed, then 5,000 FeOOH groups occupy a sphere 6.9-7.4 nm in diameter which agrees with information from chemical and MW determinations.

In electron micrographs taken slightly below focus, the iron core of ferritin appears in a variety of forms; four dense regions, a ring, a horseshoe and as a pair of parallel lines (Farrant, 1954; Muir, 1960).

1.4.4 Haemosiderin

Haemosiderin is granular, containing clusters of iron hydroxide units mixed with apoferritin.
The total content of ferric iron may reach 45% by weight (Harrison, 1964). It is thought to be formed by the same polymerisation method as ferritin, but the iron micelles become exceptionally large and cross-links occur uniting several ferritin molecules (Granick, 1949). Haemosiderin is in RES of spleen and liver (Schubart, 1966) and has been extensively studied by Bessis & Breton-Gorius (1957; 1959) and Richter (1957; 1958a,b; and 1959).

Haemosiderin aggregations are brown, Perls' Prussian Blue positive and sometimes as large as 3 μm. In EM, particles within deposits are found ranging from 3–8 nm (Richter, 1957). Some deposits are relatively homogeneous and surrounded by membranes (Richter, 1957; Bessis & Breton-Gorius, 1959), which Richter has termed siderosomes. These often resemble mitochondria with cristae, and Bessis & Breton-Gorius (1957) and Bessis (1959) report iron aggregation in mitochondria; rats are given ethionine or haemoglobin, and haemosiderin, delimited by membranes, is found in hepatic parenchymal cells, endothelial cells and liver macrophages, as well
as in renal tubular cells and in the spleen (Richter, 1959).

Siderosomes are often observed to show disruptive changes; this may mean haemosiderin is catabolised within them, although Richter (1959) points out that a cell may contain large dense aggregations inside as well as outside membranous bodies which is more likely to mean aggregates are liberated from the bodies where they are formed and the variations shown are different developmental stages. Haemosiderin particles, as well as having the appearance of ferritin also exhibit the same lattices as ferritin crystals (Richter, 1958a). At times the subunits of ferritin and haemosiderin are indistinguishable (Richter, 1958b). He shows a unit micelle smaller than any previously seen which may be a constituent of the larger micelles, which he saw in the ferritin cores.

1.4.5 **Iron administration**

Iron deficiency anaemia is a widespread condition which usually responds well to oral iron therapy. But, owing to some patients' failure to absorb from the intestine or their
unco-operative attitude, many patients are now given intramuscular or intravenous single total doses of iron. Intramuscular are the easier of the two injections to do; there is wide variation in uptake from the injection site between species, which can be attributed to structural variations (Golberg, 1960). Muscle repair following one large dose of ID is more rapid than if the same total dose is divided over several weeks. This method of administration in humans is successful, in that 90% of the dose is absorbed in 4-5 d, but there may be staining of the tissue in the area of injection. More seriously, Richmond (1959) shows sarcomata at the injection site in rats given weekly injections of 20 mg Fe as ID (80 mg Fe/Kg), but these did not develop until 11th-16th month.

Overloading experiments on animals are described by Golberg (1960) and his assessment is that ID cannot be regarded as a significant factor in sarcoma formation. Golberg, Martin & Smith (1960) suggest there may be a threshold dose below which injection site sarcomata do not
arise. Roe & Carter (1967) use ID to induce tumours at injection sites in rats and find that the tumour incidence is directly related to dosage. In their experiments, 50% of rats on 600 mg Fe total dose showed tumours, whereas only 6% had tumours after 75 mg Fe. Richmond (1959) does not report tumours in rats given 10 mg Fe as saccharated iron oxide for 17 m nor in those given the same volume of dextran. Haddow & Horning (1960) are uncertain as to whether the carcinogenic property is in the iron, or in the entire complex. However, there are no reports of iron-dextran induced cancer in man described, even though at that time iron dextran had been in use for 10 yr and in the first 3 yr 10¾ million doses were given (Cox, 1964).

Intravenous administration is a more difficult technique and as yet is not frequently used; nevertheless many workers report successful treatment, Fielding (1961); Bonnar (1967); Thaman & Dogra (1967); Will (1967, 1968) to mention only a few. ID is given in doses of 0.3 x wt in lbs x Hb% deficiency. Bonnar (1967) reports on the use of ID in obstetrics and
gynaecology. Of 100 antenatal patients in 36th wk of pregnancy, with mean Hb of 8.5 g, the mean increase in the first week was 1.67 g, and in the second 1.51 g. In 40 gynaecological patients the pre-treatment range of Hb was 5-9.6 g, and after 4 wk all had Hb over 11 g, 36 of them over 12 g. In both obstetrics and gynaecology, the rate of response is found proportional to the severity of the anaemia.

Taj-Eldin, Falaki & Rahim (1968) report ID administration to 50 anaemic children and only 9 failed to respond satisfactorily; all of these had previous infections. Nath & Omar (1967) find the haemoglobin rise in the first two weeks after administration is most marked, about 3 g/100 ml. They report in 34 cases, 17% show side reactions, with arthralgia being commonest, and general malaise, headache, thrombophlebitis and fever, with toxic reactions in 12% and death of one man whose Hb was initially 2.4%.

Pregnant monkeys are used by Cotes, Moss, Muir & Scheuer (1966) for treatment with ID by intravenous injection in doses of 32 or 100 mg
Fe/Kg wt. Most iron is seen in the RES cells, particularly Kupffer cells of liver, within 1 wk. There is some transfer of iron across the placenta to the foetus (Muir, 1966).

In any iron therapy overdosage may occur, resulting in haemosiderosis (Cappell, Hutchinson & Jowett, 1957; Golberg, 1957). This can be avoided by careful control of iron dosage.
2.0 Materials and Methods

2.1 Animals

2.1.1 Numbers

Rats used in the experiments were:

(i) EM untreated controls - 33 Wistar aged 7, 14, 21, 32, 34, and 59 wk.

(ii) EM iron treated - 10 Sprague Dawley and 78 Wistar aged 7 wk at time of injection.

(iii) Fixation tests - 65 Wistar of various ages.

(iv) Histology - 45 Wistar, same ages as those for EM.

(v) Histochemistry - 50 Wistar aged 7-10 wk and 59 wk.

(vi) Atomic absorption spectrometry - 13 Wistar aged 7 wk.

The rat-house was kept at 21°C and the rats allowed food and water ad libitum.

2.1.2 FPL 2000

The iron used was FPL 2000, a colloidal iron dextran supplied by Fisons Limited - Pharmaceutical Division. FPL 2000 is a sterile
solution of an iron-dextran complex containing 20% iron (200 mg Fe per ml). It is prepared by neutralising ferric chloride with alkali in the presence of a dextran derivative formed by treating a dextran of weight-average molecular weight around 5000 with potassium cyanide. After excess salt is removed, heating and subsequent concentration produce a stable, clear, dark brown solution.

The preparation has a pH of 5.2-6.5 and is isotonic with body fluids, containing as it does a small quantity (1.0-1.5%) of sodium chloride. It is of low viscosity (less than 30 centistokes at 25°C) and of low toxicity: the LD$_{50}$ on intravenous administration to mice is greater than 3000 mg iron per Kg body weight.

The iron was injected into the tail veins of 7 wk-old rats of both sexes in single doses of 20, 60, 400 and 500 mg Fe/Kg body weight. The small doses were administered using a micrometer syringe.

2.1.3 Anaesthesia

Animals used for histology and some for histochemistry were killed using CO$_2$. Those
for EM were injected intraperitoneally with sodium pentobarbitone in doses of 4 mg/100 g body weight.

2.2 **EM Techniques**

2.2.1 **Fixation**

(i) **Immersion.** A 1 mm slice from a liver lobe was removed and cut into 1 mm cubes. These were immersed in fixative for various times.

(ii) **Perfusion.** The apparatus used to perfuse the livers was as shown in Fig 2.

Syringes were 30 ml disposable, the left containing pre-fixation wash and the right the fixative. The central column could be varied to obtain different perfusion pressures. The cannula was of flexible polythene, with 2 mm internal bore.

A cannula was tied in the hepatic portal vein, pre-fixation wash forced through and the caudal vena cava simultaneously cut. When the liver blanched, showing that most of the blood was washed out, the fixative was introduced and continued flowing until the liver appeared fixed.
For aldehyde fixatives this was when a hard, waxy appearance was noted, and for osmium fixation when the tissue became black. 1 mm cubes were then immersed as in the previous method.

When aldehyde fixation was used, the tissue was washed in three changes of buffer then post-fixed in OsO₄ for one hour before processing.

See Table 3 for different fixatives used.

2.2.2 Processing

(i) Washing. In early work, tissue was washed in 10% ethanol at 20°C for 1–24 hr. This was found to be damaging to the tissue, so was discontinued.

(ii) Dehydration. In early work, dehydration was in 100% ethanol for 3 x 30 min at 20°C. Clearing was in propylene oxide for 30 min. Later this method was revised. Immediately following osmication, or a buffer wash after aldehyde, the tissue was dehydrated in 40%, 50%, 70%, 90%, 100% ethanol for 5 min each at 4°C, followed by 100% ethanol for 10 min at
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<th>Pre-fixation wash</th>
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<td>3% glut (B) in 1/3 strength Millonig</td>
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<td>Postfix 1% OsO₄ in H₂O</td>
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**Pre-fixation wash**

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**Solution**

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<td>8.8</td>
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<tr>
<td>23)</td>
<td></td>
<td>1% OsO₄ in 0.17M cacod</td>
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**NOTES:**
1. Sabatini, Miller & Barrenett (1964)
2. Caulfield (1957)
3. Luft (unpublished, 1965)
4. Holt & Hicks (1961a)
5. Ito & Karnovsky (1969)
6. Karnovsky (1965)
7. Luft (1959)
8. Millonig (1961)
9. Sjöstrand (1967)

**ABBREVIATIONS:**
- glut B - bench glutaraldehyde
- " S - Sigma "
- " T - Taab "
- " TAC - "

purified by shaking x 3 with activated charcoal (Fahimi & Drochmans, 1965)
<table>
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<td>glut</td>
<td>L-glutaraldehyde</td>
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<tr>
<td>Ladd</td>
<td>Ladder</td>
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<tr>
<td>glutaraldehyde</td>
<td>glutaraldehyde</td>
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<tr>
<td>cacod</td>
<td>sodium cacodylate</td>
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<tr>
<td>form</td>
<td>formaldehyde</td>
</tr>
<tr>
<td>sucor</td>
<td>sucrose</td>
</tr>
<tr>
<td>gluc</td>
<td>glucose</td>
</tr>
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<td>anhyd</td>
<td>anhydride</td>
</tr>
<tr>
<td>phos</td>
<td>phosphate</td>
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20°C, then cleared in propylene oxide for 20 min.

2.2.3 Embedding

Araldite was prepared as follows:-

(A) \( \frac{1}{2} \) lb Araldite resin CY212 + \( \frac{1}{2} \) lb Hardener HY964 mixed thoroughly and stored at 20°C.

(B) 5 ml Accelerator DY064 + 20 ml Dibutyl phthalate mixed and stored at 4°C.

(C) 19 ml of (A) + 1 ml of (B) mixed by continuous rotation for several hr before use.

The Araldite mixture at 20°C was poured into shallow dishes, or BEM capsules and the cubes of tissue immersed and set aside overnight. They were transferred to fresh Araldite mixture at 60°C and allowed to polymerise at 60°C for at least 48 hr. After polymerisation tissue blocks were sawn out and mounted in sealing wax on 8 mm dowel rods. They were trimmed and sectioned using either LKB Ultratome I or Huxley Ultratome. 1 \( \mu \)m sections were stained with toluidine blue and examined, and selected areas then sectioned at 60-70 nm, mounted on Athene 483 grids, stained and examined in an
AEI EM6B. Micrographs were taken using Ilford Special Lantern Contrasty 3/4" sq plates, and developed in Ilford ID2 diluted 1 + 4 for 4 min and fixed in Hypam diluted 1 + 4 for 10 min.

2.3 Staining
2.3.1 Lead citrate
   In early work this was made by dissolving a few grains of lead citrate and one pellet (0.15 g) of NaOH in 20 ml freshly boiled distilled water. Grids were floated on this, sections down for 2 min, then washed in NaOH made from one pellet in 100 ml boiled distilled water, and rewashed in distilled water.

2.3.2 Uranyl acetate I
   This was used after the lead stain. Saturated uranyl acetate in 50% ethanol was centrifuged and decanted. Grids were immersed for 10 min then washed for 20 sec in 50% ethanol. It was found that better contrast was obtained using the following methods.

2.3.3 Uranyl acetate II
   Uranyl acetate in 1% acetic acid was kept in the dark for several weeks, and well shaken
every few days until it was saturated. The supernatent was decanted into a flat dish and allowed to begin to crystallise. Large crystals were removed and placed on a rigid polythene platform on a watchglass in a covered petri dish. Each crystal was covered by a large drop of uranyl acetate and the watchglass was surrounded by the same solution. This dish was kept in the dark and replenished when necessary. Staining was done by placing the grid, sections down, on the uranyl acetate for 10 min, then washing in a stream of distilled water for 30 sec. This was followed by lead staining.

2.3.4 Lead citrate (Reynolds, 1963)

After uranyl acetate stain sections were stained in lead for 2 min then washed in a stream of distilled water for 30 sec.

2.4 Histology

3 mm slices of liver were fixed at 20°C for 16-24 hr in fixative of:-

saturated aqueous HgCl₂ - 9 parts
40% formaldehyde - 1 part

They were dehydrated and cleared in an Elliott
Tissue Processor, embedded in paraffin and cut at 5 μm. Before staining, sections were put through 0.5% iodine in 80% ethanol for 5 min, rinsed in water and put through 3% aqueous sodium thiosulphate for 1 min. They were then stained according to methods detailed in Culling (1963) and mounted in XAM.

2.4.1 Haematoxylin and Eosin

Harris haematoxylin for 4 min and aqueous eosin for 2-4 min.

2.4.2 P.A.S.

2.4.3 Perls' Prussian Blue Reaction

To show a blue reaction over FPL 2000, it was necessary to stain in potassium ferricyanide at 20°C for 20 min, then transfer to fresh solution for the same time. Counterstaining was with neutral red.

Perls' Prussian Blue reaction was also done on some Araldite sections using the method of Tanaka and Beschauer (1969).

Slides were photographed using a Leitz Orthomat with Kodacolor X ASA.80 film for colour prints.
2.5 Histochemistry

2.5.1 Light histochemistry

3 mm slices of liver were fixed in formol-calcium at 4°C for 16 hr:

- 40% formaldehyde 10 ml
- distilled H₂O 90 ml
- CaCl₂ anhydrous 1 g

This was replaced with acacia sucrose at 4°C:

- sucrose 30 g
- acacia 1 g
- distilled H₂O 100 ml

It was stored in this until required for histochemical reactions.

8 um sections were cut on a freezing microtome and floated onto slides coated with a dry film of a mixture of equal parts of 1% gelatine and 2% formaldehyde. After drying in air for 2 hr they were used for Gomori and Barka reactions to show acid phosphatase.

(a) Gomori (1950) reaction.

Slides were incubated at 37°C for 60 min then treated with H₂S in water.

They were mounted in glycerine jelly.

(b) Barka reaction (Barka & Anderson, 1962).
Slides incubated at 20°C for 23 min.

2.5.2 **EM histochemistry**

Aldehyde fixation was used, adapting methods of Holt & Hicks (1961b), Goldfischer, Essner & Novikoff (1963), Sabatini, Miller & Barrnett (1964), Janigan (1965) and Lazarus, Volk & Barden (1966).

2 mm x 1 mm² blocks of liver were fixed by immersion after perfusion with 2.5% glutaraldehyde (Ladd) in 0.1M cacodylate at pH 7.4 at 4°C for 3-4 hr. Another fixative used was 10% formaldehyde in 0.1M cacodylate containing 1% CaCl₂·H₂O at pH 7.4 at 4°C for 6-7 hr. All the tissue was washed three times and stored in 0.3M sucrose in 0.1M cacodylate at pH 7.4 and 4°C.

The thinnest tissue obtainable from free-hand razor cuts of these blocks was incubated in Gomori substrate containing 7.5% sucrose and 1% formaldehyde at either pH 5.0 or 6.2 at 37°C for 15 min. It was washed three times in 0.05M acetate containing 7.5% sucrose and 4% formaldehyde at suitable pH at 4°C. Controls were done using 4% formaldehyde as the incubating
substrate. Washed slices were postfixed in OsO₄ for 1 hr and processed as other EM tissue, with only 2 min in each ethanol, and propylene oxide for 5 min.

2.6 Atomic absorption spectrometry

Three normal male Wistars, aged 7 wk were intravenously injected with ID (500 mg Fe/Kg). At 10 min, 20 min, 1, 2 and 4 hr, 1 d and 2 d after injection, 0.05 or 0.1 ml blood samples were taken and treated with heparin. These were diluted 10 times with 0.9% saline and centrifuged to remove the erythrocytes, leaving a plasma supernatant. For convenience, a further three animals were injected in order to obtain 16 hr samples. These six animals constitute Expt 1. A repeat Expt 2 was done with another seven animals.

The iron levels were measured by atomic absorbometry, using a Hilger and Watts 'Atompek'. The plasma samples were further diluted by trial and error to bring the iron levels within the range of the instrument (0 to 20 ppm approximately). Results were calculated
using a standard curve of suitable diluted samples of FPL 2000. Control experiments were performed showing that the diluted plasma itself made no significant contribution to the iron levels, and did not interfere with the latter determinations.
Results

Micrographs are inserted after the references.

3.1 Normal sinusoidal lining cells

Micrographs showing normal Kupffer cells after staining with H & E, Prussian Blue\textsuperscript{Reaction}, PAS and toluidine blue are shown in Fig. 3. Fig 4 shows the normal distribution of acid phosphatase in tissue of control rats stained by Gomori & Barka methods. The Gomori technique produces brown reaction product in granular form in parenchymal cells, particularly in the peribiliary areas. In some Kupffer cells there are very dark brown granules and difficulty is found in distinguishing whether this is reaction product, or phagocytosed material. It appears darker than the parenchymal cell reaction product, but this technique is known to present some artefacts, so not too much dependence must be put on it.

The Barka technique often proved difficult to photograph, because of the delicate gradations in colour. It shows a similar
distribution of acid phosphatase in parenchymal cells, with only a small amount of reaction product over an occasional Kupffer cell.

3.1.1 Cell shape

The sinusoidal lining cells of normal rat liver are pleomorphic and so their section profiles show even greater diversity. A cell, which would be typical of endothelium in other tissue, is shown in Fig 5. Other cells have long thin extensions (Figs 6, 7), or are long and broad (Fig 6). These cells usually line the sinusoid forming a wall in close apposition to the microvilli of the parenchymal cells. Sometimes, endothelial cells are found apparently bridging a sinusoid (Figs 8, 9, 16). Large rounded cells are frequently found (Figs 9, 14, 16) and occasionally a large cell appears to fill the sinusoid (Fig 19). This last type may be due to a tangential section through an edge of a sinusoid.

The size of sinusoid lining cells is very variable and there is no virtue in measuring them in random sections; for such measurements to be useful, whole cells would have to be serially
sectioned, which has not been attempted in this study.

3.1.2 Cell surface

Their surface, too, is very variable: the very thin cells, or long thin extensions of other cells (Fig 6) normally have smooth luminal and abluminal surfaces. Sometimes coated vesicles are seen on the luminal surface (Figs 7,10) and in some cells many coated vesicles are seen immediately under the cell surface (Figs 6,7,11). Less frequently, coated vesicles are seen on an abluminal surface (Figs 7,8,10,12).

The bigger cells may have either a smooth or tortuous luminal surface, sometimes with coated vesicles. The abluminal surface is normally smoother (Figs 11,12,14,15,20). A frequent observation is that the luminal surface has a quantity of flocculent material associated with it which is less often seen at the abluminal surface (Figs 10,12,13,14,16). It is also noticeable that the plasma membrane at the luminal surface has a less sharply defined appearance than at the abluminal surface.
(Figs 10,11). Cells bridging sinusoids have a tortuous outline over the entire cell (Figs 8,9,16). Very often, those cells with tortuous outlines contain phagosomes, implicating the cytoplasmic extensions in phagocytosis (Figs 9,14-16,19,20).

Some lining cells have many extensions from their luminal surfaces close to an area of micropinocytosis vermiformis (Figs 12-14). This shows circular and worm-like profiles with a dense core, not extending to their rounded ends. The diameter is approximately constant, ranging from 0.1 μm - 0.15 μm. There is no evidence of association between these bodies and coated vesicles or any other organelle. Those cells with micropinocytosis vermiformis usually also contain phagocytosed material in the same section. When micropinocytosis vermiformis is present there is often a thrombocyte lying close to that part of the membrane (Figs 12,13). This is regarded as more than coincidence, as they are never seen at other times.

Many lining cells contain phagocytosed
material. Without electron histochemical studies, it is not possible to say which vacuoles are phagosomes, and which secondary lysosomes. The Gomori technique (Fig 4) suggests that there is acid phosphatase activity in the control rat Kupffer cells, but in the same tissue only very occasionally is any reaction product shown in these cells by the Barka method.

3.1.3 **Cytoplasmic matrix**

The matrix of all the lining cells is granular, and occasionally fine filaments are observed. It is noted that their cytoplasmic matrix is never as dense as that of adjacent parenchymal cells.

3.1.4 **Organelles**

Lining cell nuclei may be oval (Figs 6,11), round (Figs 9,15), or irregular in section (Fig 8). In those cells with an oval nucleus, it almost fills the width of the cell, with thin extensions of cytoplasm on either side. With a nucleus of another shape, there is more cytoplasm surrounding it. This may be a sectioning effect, with those showing an oval
nucleus having been cut in the widest part of an ellipsoid nucleus. The chromatin is frequently clumped around the nuclear envelope (Figs 6, 8, 9, 11, 19), or may be more evenly distributed (Fig 15). Both nucleoli (Fig 9) and sphaeridies (Fig 8) are observed. Nuclear pores, 0.1 μm in diameter, are shown in Figs 17-18.

There are numerous mitochondria in lining endothelial cells. The sizes of their profiles vary, but they appear to be smaller than those in parenchymal cells. They have round, long or irregular shapes (Figs 8, 9, 15, 16). In many of the cells shown Golgi membranes are seen, often near the nucleus (Figs 9, 11), and sometimes lying within an indentation into the nucleus (Fig 8). Occasionally they are found at the periphery of the cell (Fig 21). Those cells containing phagosomes frequently show more areas of Golgi membranes than other cells, sometimes with as many as 6 areas of Golgi within a small area of cytoplasm (Figs 9, 12, 16). Always near these prolific Golgi areas are very large numbers of Golgi vesicles.
A centriole is seen occasionally in a Kupffer cell (Fig 16). Others are seen in Kupffer cells of iron-treated animals.

Many Kupffer cells contain free ribosomes and most sections contain a few cisternae of RER (Figs 16-19). Many also contain SER (Fig 9) and some cells have membranous transitions from RER to SER (Figs 7,9,20). Glycogen is not seen in any of these cells. Some cells have small, round electron dense bodies which may be lysosomes (Figs 7,8,9,12-16,19,20). Residual bodies, some containing myelin figures, are sometimes seen (Figs 12-14).

Lining cells may overlap, with a gap of less than 20 nm between the apposing membranes (Fig 14). Where there is no underlying cell, a pore of very variable size may exist (Figs 6,7).

3.1.5 Ageing

The only difference seen in Kupffer cells from older rats, those of 59 wk, is that they are frequently almost full of large round vacuoles containing flocculent material mixed with dense material of the same electron-density as erythrocytes (Figs 21,22).
These are also seen in young rats, but only very occasionally (Fig 15), and seldom more than one in any cell.

3.2 Ultrastructure of Iron Dextran

The ID used is a colloidal solution whose particles have a crystalline shape; some are needle-like while others are more ovoid. The mean length of the crystals is 34 nm and the width varies from 4 nm - 8 nm. When viewed at low power in tissue, they appear very electron-dense. When higher power is used on isolated ID (Fig 23a), the crystals appear to have a granular structure, with lighter and darker areas in them. Some particles are much lighter than others. When using the JEM 100U, longitudinal lines with 1 nm periodicity are seen (Fig 23b).

3.3 Atomic absorption spectrometry

Results obtained from the rats injected with 500 mg Fe/Kg are as shown in Table 4. The 20 min result in Expt 1 is regarded as suspect, but the rest of the results show that
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<td>1</td>
<td>4 hr</td>
<td>570</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>16 hr</td>
<td>336</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1 d</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2 d</td>
<td>5.5</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>10 min</td>
<td>805</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>20 min</td>
<td>740</td>
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<tr>
<td></td>
<td>1</td>
<td>1 hr</td>
<td>740</td>
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<tr>
<td></td>
<td>1</td>
<td>2 hr</td>
<td>680</td>
</tr>
<tr>
<td>Experiment</td>
<td>Animal</td>
<td>Time Period</td>
<td>Mg non-Hb Iron/100 ml Blood</td>
</tr>
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<tr>
<td>2</td>
<td>1</td>
<td>4 hr</td>
<td>585</td>
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<tr>
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<td>2</td>
<td></td>
<td>625</td>
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<td></td>
<td>3</td>
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<td>630</td>
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<tr>
<td>4</td>
<td>1</td>
<td>16 hr</td>
<td>307.5</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td></td>
<td>282.5</td>
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<tr>
<td></td>
<td>3</td>
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<td>4</td>
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<td>302.5</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>1 d</td>
<td>212.5</td>
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<tr>
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<td>2</td>
<td></td>
<td>277.5</td>
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<tr>
<td></td>
<td>3</td>
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<tr>
<td>8</td>
<td>1</td>
<td>2 d</td>
<td>8.75</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td></td>
<td>9.75</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td>5.50</td>
</tr>
</tbody>
</table>
the ID disappears from the blood slowly at first, with about 75% ID still in the blood after 4 hr and total clearance taking place between 24 and 48 hr. See Fig 24.

3.4 Iron-treated tissue

The Perls' Prussian Blue reactions do not show very much variation except when a large period of time has elapsed. So four representative sections are shown as Fig 25. The PAS stain was capricious so only those sections stained in the same batch of Schiff's reagent will be used for comparison.

The results of the acid phosphatase reactions are summarised in Table 5 and representative micrographs are shown in Figs 26 and 27. Tissue from the same specimen has been used for the two techniques to attempt to eliminate errors which are frequent in acid phosphatase incubation.

3.4.1 Uptake of ID

The uptake of ID, as shown by electron microscopy takes place within 1 d of administration, starting within 10 min of
### Table 5

Acid Phosphatase in Kupffer Cells as Shown by Gomori (1950) and Barka & Anderson (1962)

<table>
<thead>
<tr>
<th>Dose (mg Fe/Kg)</th>
<th>Time after dosage</th>
<th>Sex</th>
<th>Gomori</th>
<th>Barka &amp; Anderson</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 400</td>
<td>-</td>
<td>M</td>
<td>599</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2 hr</td>
<td></td>
<td>587</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>17 hr</td>
<td></td>
<td>584</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>1 d</td>
<td></td>
<td>578</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>2 d</td>
<td></td>
<td>579</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>4 d</td>
<td></td>
<td>574</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>8 d</td>
<td>F</td>
<td>575</td>
<td>no result</td>
</tr>
<tr>
<td></td>
<td>14 d</td>
<td>M</td>
<td>571</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>16 d</td>
<td></td>
<td>589</td>
<td>++</td>
</tr>
<tr>
<td>Control 500</td>
<td>1 yr</td>
<td></td>
<td>597</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td></td>
<td>591</td>
<td>+</td>
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<td>20</td>
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<td>593</td>
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<td>592</td>
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<td>20</td>
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<td>594</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>1 yr</td>
<td></td>
<td>596</td>
<td>+</td>
</tr>
</tbody>
</table>

Parenchymal cells did not show any significant increase or decrease in quantity of reaction product. 

No acid phosphatase demonstrable -

Small number of granular deposits +

Large number of granular deposits ++
injection and proceeding slowly at first, then after about 1 hr becoming more rapid. 

a) 10 min reaction  

Prussian Blue shows intense blue between the erythrocytes in the veins and arteries, due to the ID mixed in the plasma. There is no reaction in any of the liver cells (Fig 25a). PAS shows a small amount of positive material; both these are very similar to 7 wk control animals. 

Electron micrographs show distended micropinocytosis vermiformis containing a few crystals of ID on the luminal surface of a Kupffer cell (Fig 28). In the vicinity of this area a thrombocyte is seen in the sinusoidal lumen. Immediately below the cell surface are several small vesicles containing ID and further into the cell are larger vesicles containing ID and other less electron-dense material. The largest vacuoles in the cell have a tortuous outline and inside the membrane is material of similar density to that in the centre of the worm-like bodies. Fig 29 shows another Kupffer cell with a coated vesicle
containing ID immediately below the abluminal surface. Towards the abluminal surface is micropinocytosis vermiformis of an unusual form; it is forming a figure-of-eight, and beside that a figure-of-six, with ID contained within the distension. It is unusual to find micropinocytosis vermiformis on this surface, and this was the only time it was observed in this study. Other vacuoles containing ID, mixed with various contents, are also seen.

The typical appearance of ID in a cell at 10 min is seen in Fig 30. The crystals are loosely dispersed within a large vacuole. Other smaller vacuoles are seen nearer the cell surfaces. The cell in this figure shows the fine filaments described in control livers. ID is not found in any parenchymal cells, but is observed within intercellular spaces (Fig 31).

b) 20 min

This is very similar to 10 min. The ID is found entering the cell by micropinocytosis vermiformis (Fig 32) and is in secondary lysosomes formed from autophagic vacuoles (Figs 33, 34). It is also entering in coated vesicles (Fig 33);
these appear to take only one or, at most, very few crystals each. The ID is very loosely packed in the lysosomes. In Fig 34 there is a vesicle containing ID inside a vacuole which is curved around another vacuole, possibly about to coalesce. Cytoplasmic filaments are seen in Fig 34.

c) 2 hr

Acid phosphatase is very similar to that in control tissue, except that Kupffer cells now contain ID which is mixed with reaction product, seen most distinctly by the Barka method (Figs 26a, 27a).

ID is observed entering the Kupffer cells via deep invaginations into the membrane (Figs 35, 36). These are not micropinocytosis vermiformis. The Kupffer cells now contain many vacuoles of ID, some round in profile, but already many have elongated or dumbbell-shapes. It is much more closely packed than previously and each vacuole contains some granular electron-dense material embedded in the ID. This is interpreted as undispersed enzymes derived from primary lysosomes. Small
vesicles of similar density are found around these vacuoles and are interpreted as primary lysosomes.

In Figs 37, 38 unusual shapes of ID vacuoles are shown; Fig 37 suggests a bridge or tube between two vacuoles, while in Fig 38 the vacuole has a long twisted extension containing only a few crystals of ID.

The ID is being phagocytosed by hepatic parenchymal cells (Fig 37) and is already in some small vesicles and pericanalicular dense bodies (Fig 39). It is noticeable at this stage that there is little cytoplasmic ferritin in any of the cells.

d) 3 hr

Uptake of ID is continuing through both surfaces of lining cells and into parenchymal cells (Fig 40). The vacuoles are still loosely packed and are now showing more diversity of shape, including a V-shaped profile. They all contain considerable amounts of the dense granular material described above. In Fig 41 Golgi lamellae are shown near a phagocytic vacuole containing granular material of a similar
density to that in nearby Golgi vesicles. Vacuoles of autophagic origin in the parenchymal cell contain ID and within the same vacuole is seen ferritin (Fig 42). These are concentrated around the peribiliary area. There is still very little cytoplasmic ferritin.

e) **17 hr**

Kupffer cells now contain more PAS positive material than in controls. Both acid phosphatase reactions show similar results to 2 hr. Electron micrographs show uptake of ID in coated vesicles, one such cell contains a centriole (Fig 43). The ID contained in many large vacuoles is now closely packed and some vacuoles have taken up bizarre shapes (Figs 44, 45). Some vacuoles still show dense granular material, Fig 45, and in others it is obliterated (Fig 44).

### 3.4.2 Intracellular ID

a) **1 d**

Prussian Blue reaction shows deep blue masses in Kupffer cells, often at the periphery of lobules, but there is so much throughout the
the lobe that a pattern is very difficult to show. There is punctate blue in many parenchymal cells interpreted as vesicles of ID; these are around the peribiliary area (Fig 25b). The PAS shows more positive material than previously. Acid phosphatase does not show any change from 17 hr.

Electron micrographs are similar to those at 17 hr, with densely packed ID in many vacuoles in Kupffer and lining cells (Fig 46). The main difference is in the large amount of ID in vacuoles in parenchymal cells, taken to correspond to the punctate blue in the Perls' Prussian Blue. An increased amount of ferritin is seen in parenchymal cytoplasm and in some Kupffer cell vacuoles.

b) 2 d

Prussian Blue reaction now shows diffuse blue around peribiliary areas, with PAS and acid phosphatase similar to 1 d.

Kupffer cells show very large aggregations of ID in some cases and this is now liberally mixed with ferritin (Fig 47). The large vacuole in Fig 47 may have been formed from
many smaller vacuoles since it contains clumps of ID in rounded shapes, but no internal membranes are seen. Beside the vacuole is a small siderosome.

Fig 48 shows residual bodies and vacuoles of ID beside a bile canaliculus containing ID. This may be following cellular defaecation, but the possibility of an artefactual rupture along the canaliculus should be considered.

c) reaction

Prussian Blue is similar to 2 d as are the acid phosphatase specimens. PAS shows a further increase in positive material.

Many Kupffer cells have large tortuous vacuoles of ID and there is more ferritin and less ID than at earlier times (Fig 49). In this micrograph the ID around the periphery of the vacuole seems to be unadulterated while that further into the vacuole is mixed with ferritin. There is also ferritin in the cytoplasm of the Kupffer cell. In other cells the intact ID is also in patches, not necessarily with the same distribution as shown. ID is again seen in a bile canaliculus.
d) 8 d

Reaction

The Prussian Blue reaction remains similar to the two previous specimens, but both acid phosphatase specimens show more diffuse reaction product in parenchymal cells, with much reaction product over Kupffer cells. There is very much more PAS positive material than in other specimens.

In Kupffer cells, there are seen vacuoles containing in the plane of section only ferritin without any ID; these are called siderosomes. They remain in very contorted shapes. In other Kupffer cells, some vacuoles, similar to those in 59 wk control rats, contain flocculent material mixed with ferritin (Fig 50). Many very tortuous ID-filled vacuoles remain. The cytoplasmic ferritin is now noticeably more concentrated in parenchymal than in lining cells, bile canaliculi contain ID as before.

e) 12 d

All the light microscope specimens are similar to those at 4 d. Some Kupffer cell vacuoles contain ID mixed with iron in smaller particles (<5 nm) than ferritin (Fig 51) which
sometimes align like a string of beads. Ferritin is seen around the edge of the vacuoles containing patches of ID. Fig 52 shows siderosomes and ID in vacuoles in a Kupffer cell. Counts of cytoplasmic ferritin on single micrographs show a parenchymal to Kupffer cell ratio of 5:2.

f) \textbf{16 d} reaction

Perls' Prussian Blue is similar to those described previously, except the punctate blue is now replaced by diffuse blue (Fig 25c). Acid phosphatase distribution is shown in Figs 26b, 27b. Reaction product is mixed with ID in Kupffer cells, as well as being in parenchymal cells. The Kupffer cell vacuoles show mixed ID and ferritin and smaller iron particles (Fig 53), with ID around the periphery.

g) \textbf{22 d} reaction

Prussian Blue still shows large blue masses over Kupffer cells, but now the diffuse blue is more widely distributed throughout the lobe. There is still much ID in Kupffer cells. Some of these cells show more Golgi lamellae than do controls. The vacuoles are often very tortuous
shapes, with suggestion of fusion between several vacuoles (Fig 54). There is still much ID in parenchymal vacuoles and much more ferritin in parenchymal than Kupffer cells. ID is again found in a bile canaliculus.

h) 7 wk

500 mg Fe/Kg

Prussian Blue reaction shows many blue masses over Kupffer cells, though less than before, and much more diffuse blue throughout the tissue. Kupffer cells in electron micrographs still show much ID, but a higher proportion of ferritin is seen than previously (Fig 55). Cytoplasmic ferritin concentrations have been compared by counting the number of ferritin profiles in adjacent areas in both parenchymal and Kupffer cells; their ratio is 5:1.

60 mg Fe/Kg

Only a few Kupffer cells have blue masses in Prussian Blue reaction and there is no diffuse blue in the parenchyma. Some Kupffer cells' vacuoles show patches of ID and between these is a little ferritin (Fig 56). There are less vacuoles of both ID
and ferritin than in the 500 mg Fe/Kg specimens. Cytoplasmic ferritin is also reduced, as are parenchymal siderosomes. Some vacuoles like those in 59 wk controls are seen in Kupffer cells.

20 mg Fe/Kg

Very occasional Kupffer cells show a little blue, but most tissue is devoid of blue, like the controls. Some Kupffer cells with ID and ferritin, but much less than with higher doses of iron. Some cells have vacuoles containing ferritin mixed with flocculent material like those in 59 wk control (Fig 57). Tissue from female rats was examined, without any significant differences being found.

i) 14 wk

500 mg Fe/Kg

Less frequent blue masses over Kupffer cells than in 7 wk specimen, and very much more diffuse blue still throughout the tissue.

There is still much iron in Kupffer cells, but only a few crystals of ID are observed (Fig 58). Many Kupffer cells only have a few siderosomes (Fig 59). There is still more cytoplasmic ferritin in parenchymal than in
Kupffer cells.

60 mg Fe/Kg

There is very little ID in Kupffer cells and no diffuse blue in parenchyma. Some Kupffer cells have vacuoles like those in 59 wk control.

20 mg Fe/Kg

Occasionally a trace of blue is seen in a Kupffer cell, but none elsewhere.

j) 27 wk

500 mg Fe/Kg

Less blue masses over Kupffer cells than at 14 wk and only a little diffuse blue throughout the tissue in Perls' Prussian Blue reaction. Many residual bodies and siderosomes in parenchymal cells, and much cytoplasmic ferritin. Kupffer cells are similar to high dose 14 wk specimens.

60 mg Fe/Kg

Very few blue masses over Kupffer cells, no diffuse blue in parenchyma.

20 mg Fe/Kg

Perls' Prussian Blue reaction is the same as controls. What vacuoles remain in Kupffer cells usually contain only ferritin (Fig 60), but sometimes
a trace of ID is detected (Fig 61). There is not any ID seen in later specimens. Many cells are like 59 wk controls.

k) 52 wk

**500 mg Fe/Kg**

Prussian Blue shows many small blue aggregations over Kupffer cells, and still some diffuse blue (Fig 25d). There is more acid phosphatase throughout than in 59 wk control, with much over Kupffer cells (Figs 26c, 27c).

Many ferritin or haemosiderin aggregates are still in Kupffer cells, (Figs 62-69), with some showing iron around the edges when the rest of the vacuole is almost empty (Fig 67). The Golgi area is often prominent in the Kupffer cells (Figs 68, 69). Usually the cytoplasmic ferritin in parenchymal cells and Kupffer cells appears equally concentrated (Figs 64, 70), but where Kupffer cells have little iron in vacuoles, there is less cytoplasmic ferritin.

**60 mg Fe/Kg**

Prussian Blue is like 59 wk control. Acid phosphatase is very diffuse in parenchyma, and much still seen over Kupffer cells. Many large
vacuoles are seen like those in controls (Fig 71). This cell also has much SER in the form of tubules.

20 mg Fe/Kg

All light microscope specimens resemble 59 wk controls. Residual bodies are seen in Kupffer cells, alongside micropinocytosis vermiciformis (Fig 72). There is little background ferritin in the serial sections (Figs 73, 74) in either Kupffer or parenchymal cells.

In the 500 and 60 mg Fe/Kg a sexual difference appears to be present, in that female rats contain more Prussian blue masses in all and deeper staining than their male counterparts.

3.5 EM histochemistry

Tissue from rats injected with 0.5 ml ID 14 d previously, incubated at pH 6.2 is shown in Figs 75-78. Fixation is very poor, but parenchymal cells show acid phosphatase in peribiliary areas, as expected from light microscopy. Fig 78 shows a Kupffer cell containing ID, but it is impossible to distinguish reaction product from the dense ID.
3.6 Fixation tests

Since the micrographs in this thesis are being used for comparison it has been decided to use those obtained from tissue fixed by only two of the methods tried. The most consistent, best preservation of both Kupffer and parenchymal cells is shown by 1% OsO$_4$ in 0.17M cacodylate, used at pH 7.4, 0°C and perfused for 3-5 min at 8.8 mm/Hg pressure. Other fixatives were unsatisfactory for various reasons.

The aldehydes were tried first and the tissue fixation appeared satisfactory when viewed at low magnification, but at higher power precipitate was seen in the tissue, which was interpreted as being the result of interaction of glutaraldehyde impurities in the tissue reacting with osmium during postosmication. Even prolonged washing did not prevent this occurring. Various brands of glutaraldehyde were tried and only after a very long time was a brand found which did not have this reaction. Glutaraldehyde used without postosmication did not preserve membranes with any degree of clarity and in all cases Kupffer cell membranes
were often ruptured.

Formaldehyde was tried and found to preserve the cells well, except Kupffer cell membranes which were often ruptured, and the lipid in the tissue was extracted. Acrolein was difficult to use under our laboratory conditions and the preservation was not as good as with other aldehydes.

It was found that fixation without a pre-fixation wash was more satisfactory, probably due to osmolarity of the prefix wash being unsuitable, although it was adjusted for mammalian plasma. As Kupffer cell fixation was the problem, all but the minimum necessary solutions were perfused and although fixation was not improved, it was not worse. During the perfusion, to try to keep the normal temperature for the Kupffer cells, $37^\circ C$ had been used throughout. It was found easier and eventually produced better results to perfuse at $0^\circ C$.

Osmium perfusion was found more successful than aldehyde fixation. Various buffers were tried and cacodylate appeared to be producing
the best overall preservation. Again the pre-fixation was eliminated, the temperature kept at 0°C, and when Kupffer cell membranes were still found to be rupturing, the pressure of the perfusion was lowered to 8.8 mm/Hg. Even after this the Kupffer cell membranes were not always good, although the fixation otherwise was good.

Dehydration was done at room temperature, with any storage of tissue done in 10% ethanol, then 30 min each in 70%, 90% and 2 x 30 min in 100%. After experimenting with these, it was found that it was at this stage of the processing that the Kupffer cell damage was occurring. The dehydration was then done at 0-4°C for very much reduced times, and the Kupffer cell membranes remained intact.

So, for perfusion of rat liver, pre-fixation wash is unsatisfactory, and the best preservation is got when temperature and pressure are kept very low and post fixation washing and dehydration are reduced to a minimum.
4.0 Discussion

4.1 Normal cells

The present observations show that there is no clear distinction between sinusoidal lining cells and Kupffer cells as many previous authors have described (Zimmermann, 1923; 1928; Yamagishi, 1959; Schmidt, 1960; Tanikawa et al., 1965; Ito & Shibasaki, 1968; Wisse, 1970a, b). Their criteria for distinction are differences in morphological detail and in phagocytic ability, whereas Knisely et al. (1948), Altschul (1954) and Bloch (1955) only distinguish between degrees of phagocytic ability of these cells, and Nathan (1908) and Fawcett (1955) make no distinction. It is, however, convenient to refer to phagocytic lining cells as Kupffer cells; such cells contain ingested material.

The recent work of Wisse (1970a) has approached this problem by similar methods to those used here, but the results obtained differ in some important ways. Wisse draws attention to the different appearance of a sinusoid after perfusion compared with that following immersion.
fixation. After perfusion he finds the lumen patent and free of all flocculent material, with only an occasional blood cell present; he describes a continuous lining formed by two elements, cytoplasmic processes and sieve plates. After immersion fixation, he describes the cellular lining as "attenuated, membrane-bound pieces of cytoplasm in a catenary arrangement along the tops of the microvilli". He notes that the lining cells thus arranged have many intercellular gaps of variable width so there is free communication between the lumen and space of Disse.

Observations in this study agree with those of Wisse, except that after perfusion fixation the pictures appear to combine the features which he found by the two methods of fixation. The sinusoid is usually patent, but occasionally some blood cells and flocculent material are found within its lumen. The structure of the lining cells conforms most closely with Wisse's description of them after immersion fixation, except that some sinusoidal lining cells have fenestrations of about 0.1 μm in diameter, thus
they mimic the sieve plates of Wisse (1970a). Frequently, gaps of varying widths between lining cells allow free communication between the sinusoidal lumen and space of Disse. No other effects attributable to fixation damage are found, so these gaps are not thought to be the artefacts described by Wisse.

It is explained in the observations that many methods of fixation were tried during this study because with all except one, that is 1% OsO$_4$ in 0.17M sodium cacodylate pH 7.4 at 0-4°C (Method 23), there was damage to the lining cells. For immersion fixation, Wisse used cold 1% OsO$_4$ and for perfusion he obtained best preservation by glutaraldehyde fixation at room temperature followed by OsO$_4$ postfixation. It seems likely that the difference between Wisse's results and those described here may be due to the actual fixative. For perfusion the pressure, duration and osmolality of the fixatives are similar in both studies. The main difference lies in Wisse's method of perfusing at room temperature with glutaraldehyde.

Fixation is an attempt to preserve tissue
in a form closest to its living state, but many of Wisse's micrographs show lining cells with ruptured membranes, a fault which was found in this work after using room temperature fixatives, or glutaraldehyde. So, it seems that the micrographs obtained following fixation by the Method 23, which show well-preserved sinusoidal cell membranes, represent the sinusoidal lining cell better than those which Wisse shows after perfusion.

The same author, Wisse (1970b), has also worked on the differences between sinusoidal lining cells by studying how they respond to a large load of intravenous colloid, such as was used in this study. Wisse claims that particles stick to Kupffer cells, but not to endothelial cells and that there are no intermediate stages. This claim does not seem justified since Wisse concedes that all types of lining cell can contain particles of colloid. So, it seems more likely that he has failed to observe small amounts of adhering colloid, a conclusion which is supported by the present observations. Thus the only prudent deduction is that the surface of
some cells is more attractive than that of other lining cells, and that the rate of uptake of colloid is related to these surface phenomena. As Wisse's evidence for Kupffer and endothelial cells being two entirely different cell types is not impressive, it seems more likely that they are the same cell type at different stages of their life cycle or even performing the same activity at different rates.

During this study, the only differences apparent within the population of sinusoidal lining cells are that some cells are larger, contain more organelles and phagocytosed material than the other lining cells, and have a more tortuous surface (Yamagishi, 1959; Tanikawa et al., 1965); these correspond to the Kupffer cells of Wisse (1970b). It is agreed with the opinion of Wisse (1970a) that such Kupffer cells do not necessarily take much, or on occasions any, part in the formation of the sinusoidal wall. Wisse (1970a) reports junctional complexes characterised by a slight increase in electron-density of membranes and neighbouring cytoplasm although Fawcett (1955)
fails to find any junctions. In this study, junctions similar to those reported by Wisse are rarely seen. Frequently, double layers of sinusoidal lining are seen, also noted by Ito & Shibasaki (1968) and Wisse (1970a); these are due to extensions of perisinusoidal cells lying outside the lining cells.

There are several features of lining cells which have not been stressed by other authors. They are the frequency of Golgi areas, and several features of the lining cell membranes.

It has been mentioned, in the observations, that those lining cells which contain many phagosomes may have as many as six areas of Golgi membranes even within the section of a single cell. It can be supposed that the Golgi areas have some functional connection with the phagosomes. The concept that lysosomal enzymes are transported from RER, via SER, to Golgi areas where they are packaged into vesicles representing primary lysosomes supports this observation and deduction (Novikoff, 1963; Novikoff et al., 1964; de Duve & Wattiaux, 1966; Cohn et al., 1966; Allison,
1967; Dingle, 1968; Arstila & Trump, 1968; Cohn & Fedorko, 1969). It could be that after phagocytosis, for digestion of the ingested material, the cell increases its content of lysosomal enzymes and that it does so by increasing production on existing ribosomes and concurrently increasing its content of Golgi membranes.

The other observations concern the appearance of the membrane of sinusoidal lining cells. The luminal membranes sometimes appear different to those on the abluminal side in that they have a fuzzy appearance. This could be due to the plane of section being tangential to the membrane, however, this seems unlikely as the entire luminal membrane can have this appearance. Dingle (1968) discusses the local alterations that allow invaginations to form and suggests that they may be due to disturbances of protein-lipid interaction. Enzymatic disturbances of the fatty acid chain of the phospholipid by phospholipidase or enzymes of the complement complex are also suggested by Dingle as possible ways of leading to diminished packing of the
lipid molecules in the membrane, thus causing lack of membrane stability. Such activity could produce changes in the appearance of the membranes.

The second observation on membrane appearance is that flocculent material is sometimes associated with the luminal surface, and is occasionally seen on the abluminal surface. This may be precipitated plasma protein, perhaps the opsonin which is necessary for the uptake of particles (Berry & Spies, 1949; Jenkin & Rowley, 1961; Hirsch, 1965a; Rowley, 1966a, b; Saba & Di Luzio, 1966; Jeunet & Good, 1967; Filkins & Di Luzio, 1968; Jeunet et al., 1968; Megirian et al., 1968; Wisse, 1970b). Another possibility is that the flocculence may be the carbohydrate which Carr (1968) correlates with particle uptake. Whatever their molecular cause, it seems likely that the membrane differences, and the flocculence, are both associated with particle uptake.

Another prominent feature of some lining cells is the presence of micropinocytosis vermiformis. Tóro et al. (1962), Orči et al.
(1967), Matter et al. (1968) report seeing this in animals following various treatments, while Rouiller et al. (1967) mention casually that these areas are also seen in control specimens. From this study it is clear that micropinocytosis vermiciformis is present in control rats and it has only been seen in cells containing phagosomes. Whether these phagosomes have arisen as a result of micropinocytosis vermiciformis is unknown; they only show the cells have been in a suitable state for phagocytosis to occur. Neither can it be said if the phagosomes have any effect on the formation of micropinocytosis vermiciformis. It may be that micropinocytosis vermiciformis is a transitory phenomenon of very short duration which occurs in any phagocytic cell. Flocculent material, similar in appearance to that described above, is associated with it and these two features may be functionally linked.

No connection between micropinocytosis vermiciformis and coated vesicles has been observed as described by Matter et al. (1968).
Perhaps not enough cells have been examined but it is also possible that this connection only occurs when the RES has been stimulated with Indian ink, tetracycline, streptozotocine injections or partial hepatectomy.

The final noteworthy features of lining cells, which do not appear to have been reported, are vacuoles containing little but flocculent, electron-dense material. Because they predominate in the oldest rats of this study, ageing is an obvious cause since they do not fit the description of any known pathological states. They contain material of similar electron-density to that of erythrocytes and, as erythrocytes are seen close to some lining cells, it is interpreted that the vacuoles contain degraded phagocytosed erythrocytes. The vacuoles may remain patent, containing waste toxic products, and be retained until the cell is full of such vacuoles and it is then destroyed. These features were noted late in this study and further work is planned to elucidate the problem.
4.2 Uptake of ID

Initial uptake of ID is shown, by atomic absorption spectrometry, to take place slowly after a large dose (500 mg Fe/Kg) with only 20% being taken up in the first 4 hr and total clearance taking place between 24 and 48 hr. This result, taken on its own, does not seem very dramatic. It should be realised that these large doses comprise a phenomenal number of particles which have been injected and only when using the EM does the full implication of the situation become apparent. Since 1 ml of FPL 2000 is estimated to contain $1.3 \times 10^{17}$ particles, the RES of each rat given a high dose of ID is ingesting $> 0.6 \times 10^{17}$ particles within 48 hr. (See Appendix I.)

There is no difference at the early time interval, e.g. 10 min, between a control liver and one after ID injection as shown by Perls' Prussian Blue. With the EM however a very exciting picture is seen. There is uptake of ID by some lining cells within 10 min of its injection. By that time the ID has travelled from the tail veins where it was injected.
through the heart and has been recirculated and phagocytosed, since already it lies within some lining cells of the liver sinusoids. The speed with which the cells have recognised the ID as "foreign substance" and have started to remove it from the blood is amazing and shows that the methods of recognition of a previously unknown foreign material and its uptake are very efficient.

By 20 min there is more ID within the lining cells. It is not only entering and being packaged into phagocytic vacuoles but it is going into apparently preformed autophagic vacuoles. Perhaps this is the cells' immediate response to a large load of colloid if it has not sufficient SER to form new vacuoles immediately (Arstila & Trump, 1968).

In livers 10 and 20 min after injection of ID micropinocytosis vermiformis is a prominent feature. In many cells, various profiles are seen, round and worm-like being the most common, and these lie mainly on the luminal aspect. Within these profiles ID lies along the central osmiophilic line, so supporting the conclusion
of Toró et al. (1962) that the central line consists of osmiophilic groups on the end of proteinous rods set at right angles to the membrane. If the flocculent material mentioned previously is concerned with sticking of particles to the membrane by either electrostatic or chemical means, then when the membrane infolds against itself, the particles sticking to it will be drawn into the cell. This may be the cells' rapid method for ingesting a large number of foreign particles.

Once inside the cell, the worm-like profiles can change into figure-of-six and figure-of-eight shapes. One end may remain worm-like while the other distends. Larger, round vacuoles are also seen, these retaining an osmiophilic flocculent lining. Presumably, these are all part of a dynamic process for transporting particles into the inner parts of the cell. In the large vacuoles with an osmiophilic edge, ID adheres to part of this edge and it is interesting to speculate on why the worm-like shape was lost. Perhaps during uptake the particles acquire a charge and so as
the membrane infolds and more particles are admitted to the micropinocytosis vermiformis cleft in sufficient quantity they repel one another and so force the membranes apart. This would account for the figures-of-six and -eight, and the round vacuoles. Another possibility is that during digestion ID exerts an osmotic effect and water is drawn in from the surrounding cytoplasm. These vacuoles are very transient, as they are not seen later than 20 min after ID administration, so they may be transferring material from the micropinocytosis vermiformis to a phagosome or secondary lysosome.

In Fig 29 micropinocytosis vermiformis is seen on an abluminal surface of a lining cell. Tóró et al. (1962), Orci et al. (1967), Rouiller et al. (1967) and Matter et al. (1968) describe it only on the luminal surface. It may have moved over from the luminal surface or it may have formed at the abluminal surface in response to the very large numbers of circulating particles, some of which penetrate into the space of Disse.

Considering the few times micropinocytosis
vermiformis has been seen under control conditions, its appearance so soon after ID administration is most striking and the interpretation put upon this is that it is the main route for the immediate uptake of circulating foreign particles of a small size. At 2 hr after ID administration in some lining cells many invaginations are seen in the cell membrane and in these many particles of ID appear to enter the cell. Some just under the cell surface have the appearance of having been pinched off forming vacuoles containing ID. These invaginations are of variable width and do not have flocculent material associated with them. They may form by a flowing of the membrane, as described by Bennett (1956), or by the "passive en-membranosis" of Tanaka (1961). Casley-Smith & Reade (1965) describe engulfment of particulate aggregates of 1 μm by pseudopodia of RE cells, but there is no evidence of that process in this study.

The third method of uptake is via coated vesicles. These are seen from 10 min after injection of ID until 17 hr, by which time they
are the only observed method of entry. They have been described as a common feature of all lining cells. This method is considered last because although the coated vesicles are so widespread and continue to form many hours after injection they never contain more than a few particles of ID. They are seen on both surfaces, but most commonly on the luminal side. By 17 hr the amount of circulating ID has dropped to less than half that injected. It may be that, as coated vesicles are such a common feature in control rats, they are the normal way of dealing with foreign particles in the blood and that the other two methods of uptake, micropinocytosis vermiciformis and invaginations, only appear in response to RES stimulation, either natural or contrived as in this study.

Daems et al. (1969) report uptake of metal colloids in coated vesicles in their review of the vacuolar system, and point out that they were first proposed for the cellular uptake of protein. It may be that metal colloids are coated with serum protein before ingestion (Hampton, 1958) which supports the view that
opsonins are important in uptake of particles (Berry & Spies, 1949; Hirsch, 1965a; Rowley, 1966a, b; Saba & Di Luzio, 1966; Jeunet & Good, 1967; Jeunet et al., 1968; Megirian et al., 1968; Pisano et al., 1968; Wisse, 1970b).

So, from these observations, micropinocytosis vermiformis and invaginations are methods for ingesting large amounts of colloid such as ID, and coated vesicles are a constant method effective when low concentrations of colloid are circulating.

4.3 Distribution of ID

4.3.1 Summary of light microscopy, histochemistry and electron microscopy

It is interesting to compare and correlate the results obtained by various methods in this study. By so doing, the use of EM as a research tool is clearly seen and its quantitative limitations are demonstrated.

As mentioned in the description of the reaction uptake of ID, Perls' Prussian Blue does not show any iron present 10 min after injection, but particles can be detected by EM. By 2 hr the first change is seen in the light microscope
following histochemical staining by Barka's method. There is a marked rise in acid phosphatase content of Kupffer cells which, when followed through to the EM, is helpful in the interpretation of micrographs. There is no increase in primary lysosomes in electron micrographs, but many vacuoles of ID are seen, so that it is possible that the increase in both is connected.

Going on to 17 hr, there is still a similar acid phosphatase result to that of 2 hr, but EM shows a variable scene. Some micrographs show Kupffer cells containing many large densely packed vacuoles of ID and others have little. Thus the unreliability of an isolated EM study is shown, as the sampling errors are great and if a few micrographs were taken to be representative, without any other evidence, a very misleading result would be obtained. However, by light microscopy it is also seen that more PAS positive material is present and remains for at least 22 d when the last PAS is recorded, so a high dextran content is indicated.

At 1 d Perls' Prussian Blue reaction shows more
iron in Kupffer cells, and in parenchymal cells punctate, blue masses. When examined by EM it is found that these are many small vacuoles containing ID, particularly in peribiliary regions. The acid phosphatase technique has reached its limitation, because it shows no change from 17 hr. It obviously is not very sensitive and its value is merely to show a substantial increase from none or only a little acid phosphatase. By EM, at this time, ferritin is shown in the parenchymal cells and Kupffer cells, but it is not sufficient to cause diffuse staining with Perls' Prussian Blue reaction.

At 2 d Perls' stain shows more diffuse blue around peribiliary areas and in EM vacuoles of ID and residual bodies are seen in these areas. What is not shown by Perls' stain at this time is that ID is in the bile canaliculi. The EM is now invaluable for showing relationships between vacuoles and the organelles within the cells.

As time after ID administration increases to 4 d the extra information got by EM increases greatly. EM is by that time showing the presence
of ID and ferritin and it is interesting to attempt to work out their inter-relationships. By 8 d parenchymal cytoplasmic ferritin is greatly increased, but there is no indication of this by Perls' stain. At 12 d there is no change in light microscope observations from those at 4 d but EM shows a very great change in the contents of vacuoles in Kupffer cells, and cytoplasmic ferritin ratios in parenchymal and Kupffer cells are estimated.

By 16 d the Perls' reaction shows diffuse rather than punctate blue. Acid phosphatase is mixed with ID in Kupffer cells and in EM the contents of Kupffer cell vacuoles are seen. At 22 d Perls' stain shows much blue over Kupffer cells and in EM much ID is seen in these cells.

Seven weeks after injection of 500 mg Fe/Kg stain there is much blue over Kupffer cells, though less than before, with diminution of ID and more ferritin in these vacuoles and more cytoplasmic ferritin. After a 60 mg Fe/Kg dose little blue staining is left at 7 wk and this is only over a few Kupffer cells. This is shown
also by EM as there is ID and a little ferritin in only some lining cells. At this time after a 20 mg Fe/Kg dose of ID even fewer cells show a little blue staining as most of the tissue looks like the control. At EM level, although there is less ID obvious than at higher dose levels, the appearance could not be mistaken for that in control specimens.

At 14 wk Perls' stain shows much diffuse blue, with less over Kupffer cells, whereas EM shows more iron still in Kupffer cells but only a little as ID particles. The 60 mg Fe/Kg results with Perls' stain and EM resemble each other and 20 mg Fe/Kg shows only a trace of blue over a few Kupffer cells with the Perls' stain. By 27 wk, there is less blue than at 14 wk in Perls' stain but Kupffer cells in EM show no difference to 14 wk. At the lower dose levels, 60 mg Fe/Kg, little blue is seen over Kupffer cells and after 20 mg Fe/Kg Perls' stain looks like control, whereas in EM ferritin is seen in vacuoles with the last observed traces of ID.

One year after a 500 mg Fe/Kg Perls' stain shows blue over Kupffer cells only in very small
aggregations but there is more acid phosphatase throughout than in control of the same age. EM shows aggregations of ferritin or haemosiderin. The 60 mg Fe/Kg is like 59 wk control in Perls' stain but acid phosphatase shows an increase whereas with 20 mg Fe/Kg all light specimens resemble controls of the same age with ferritin in low concentration showing up in the EM.

4.3.2 Comparison of distribution of phagocytosed material

The distribution of ID seen in this study corresponds to a large extent with the findings of other authors. Those who report studies after very short time intervals are Onoe & Tsukada (1964), Casley-Smith & Reade (1965), Causey (1965) and Grampa (1967). In mouse, indian ink is in Kupffer cells within 5 min (Onoe & Tsukada, 1964). Casley-Smith & Reade using thorium and carbon in rats show particles in endothelial cells after 5 min and within 15 min in large vesicles which is followed closely in these observations. Causey (1965) reports on rabbit and mouse liver, after injection of colloidal lead, and shows particles in Kupffer
and in parenchymal cells; the distribution and concentration of particles are as variable as in this study.

Grampa also notes the rapidity of colloid uptake by liver; within 15 min he reports thorium dioxide in the cells, whereas within 10 min is the time observed here. He also has a similar result for the position of the particles within the vacuoles, around the peripheral rim at first, then in a more homogeneous distribution. By 3 d he sees confluence of vesicles obscuring cellular detail. The ID in this study is also in vesicles which are apparently coalescing but in most cases cellular contents are still evident. As Grampa notes, Kupffer cells ingest more colloid than parenchymal cells.

Kluge & Hovig (1969) used rats dosed with thorotrast and after 6 and 12 hr find Kupffer cells with particles and vacuoles of thorotrast around the periphery of parenchymal cells. By 24 hr they note thorotrast in Kupffer cell lysosomes, as in this study, but their observations of mitochondrial associations is unsupported by this work. By 4 d they note
parenchymal cell particle concentration falls, but peribiliary dense bodies retain some, as do those in this work and by 7 d none is seen except in bile lumina, which reflects much faster handling than with this ID. This may be because thorotrast is a non-metabolisable substance which is consequently expelled.

Driessens et al. (1967), also using rat dosed with thorium dioxide, note some Kupffer cells loaded with particles at 1 d, whereas others had only 1-2 aggregates which is what was noted with this ID. Their results in parenchymal cells also show small irregular dense aggregates associated with mitochondria and undifferentiated ER, whereas this ID does not appear to be associated with any organelles other than in peribiliary dense bodies.

Other groups of workers, Frankel et al. (1962), Bernick et al. (1966), De Mignard et al. (1966) and Patek et al. (1967) have used injections of colloidal carbon in rats. In young rats after 1 d, Frankel et al. show a result similar to that described in this work in that carbon is in Kupffer cells throughout liver lobules. By 21 d
they see giant cells which probably correspond to the cells seen in this study with very large loads of ID. Bernick et al. (1966) report giant cells in the periphery of lobules, although this was confirmed by de Mignard et al. (1966) and Patek et al. (1967), it was not observed in ID material. Nor were non-cellular ID masses present in this tissue.

4.4 **Intracellular metabolism**

The fate of ID as observed in this study is summarised in Figure 79.

4.4.1 **Biochemical problem**

When confronted with a large load of ID the cell clearly converts it by various stages to ferritin. To do this it must activate many biochemical steps. Firstly, the dextran coat must be removed from the ID, to expose the ferric iron and render it available for ferritin synthesis. The FeOOH is in a crystal lattice formation, so it must be broken down to smaller micelles for combination with apoferritin to form the final ferritin.

Apoferritin synthesis has to be initiated or accelerated with the most probable sites
being on the ribosomes (Muir & Golberg, 1961).
The stages of synthesis of apoferritin like other proteins are three: DNA of the nucleus transmits information to a messenger molecule, m-RNA. This does not involve a change of coding and is termed transcription. The m-RNA then moves from the nucleus to the ribosomes and forms a complex with them. Amino acids are activated and amino-acyl t-RNA molecules are formed, which combine with the m-RNA ribosome complex. Peptide bonds are formed and chains of polypeptides are released from the ribosomes forming the 20 subunits of apoferritin (Harrison, 1963). Finally, when ferritin is the product, a fourth stage is included, the joining of the protein and the iron core to form the final ferritin (Harrison et al., 1967; Fischbach et al., 1969).

4.4.2 Interpretation of observations

The most striking feature of the vacuoles containing ID within 1 d after injection is that they all contain electron-dense material, amorphous in nature, mixed with the ID. It is usually in discrete areas (Fig 35) and not
diffusely scattered. Near these vacuoles small vesicles the size of Golgi vesicles and of similar electron-density to the amorphous material are seen (Figs 35, 36, 38). One is even seen inside an ID-containing vacuole (Fig 36). Acid phosphatase staining indicates an increase in lysosomal enzyme in Kupffer cells within 2 hr, so the interpretation is that these vesicles are primary lysosomes, as are the amorphous contents of the vacuoles and they have been transported from the Golgi area in response to the endocytosed ID load (Golberg et al., 1960; Meijer & Willighagen, 1961). Daems et al. (1969) show micrographs of similar vesicles fusing with phagosomes and they observe that the integrity of the lysosome is retained to some extent after entering the phagosome. This they think to be due to the lysosomal enzymes being linked within their granules to a glycoprotein matrix. They do stress that merging of primary lysosomes and phagosomes is only rarely shown in micrographs, as is confirmed in this study. These observations are in agreement with those of Ericsson et al. (1965), Straus (1967) and
The idea that lysosomal enzymes are synthesised on RER and transported via Golgi vesicles coincides with interpretations in this study, since many Kupffer cells with ID also have much RER and Golgi, although not more than the controls which have phagosomes (Novikoff, 1963; Novikoff et al., 1964; Cohn et al., 1966; de Duve & Wattiaux, 1966; Allison, 1967; Arstila & Trump, 1968; Dingle, 1968; Cohn & Fedorko, 1969).

Very little of this material, which is interpreted as being lysosomal enzyme, is seen after 1 d. It may be obscured by ID which is then tightly packed in vacuoles, or it may have diffused throughout the vacuoles. The latter seems unlikely since it is noticeable that the breakdown of ID is irregular as within one vacuole there may be a patch of ID, a patch with much ferritin but devoid of ID and another area not containing any electron-dense material. Even after many weeks, Golgi vesicles are in the cytoplasm in the vicinity of ID vacuoles. This could explain why acid phosphatase concentration
is higher in treated as opposed to control rats after 1 yr. It may be that once the lysosomal enzyme production in a cell is increased, as after ID ingestion, it remains high during the life of that cell, even though the ID is digested. The other possibility is that the cell which ingested and digested the ID has reached the end of its life, perhaps earlier than a cell in a control animal, and has been ingested by another phagocytic cell and at 1 yr the raised acid phosphatase is involved in digestion of the original cell. In both these instances it is conceivable that although the ID is not seen in the EM, the iron which is seen is not necessarily in the form for incorporation into ferritin, and lysosomal enzymes are still being used for this.

Another noticeable feature of ID vacuoles in this study is their contorted shapes. A few hours after injection round vacuoles are seen but by 1 d many tortuous vacuoles have appeared. These are either formed from coalescence of spherical vacuoles as suggested by Muir & Golberg (1961) or from the ID being taken up by
a lamellar or tubular system, such as ER or Golgi membranes. In two micrographs tubular shapes are seen. It is interesting to speculate on the suitability of RER for this job, as the membrane surrounding ID is single and observations have frequently shown alternations between RER and SER, it is possible to speculate that ID might enter the lamellae and as the membranes separate, ribosomes are removed. Since both enzymes and apoferritin are in demand at the site of ID, the thought that they would be manufactured on ribosomes further along the same tubular system is attractive. This possibility cannot be discounted but from the frequency of multiple spherical shapes seen, coalescence of simple spherical vacuoles is obviously the predominant method of formation of tortuous vacuoles. They may form in response to a very high load of colloid, as Richter (1959) and Daems et al. (1969) also report them after ID and Kluge & Hovig (1969) see them after thorotrast injections.

As lysosomal enzymes enter the ID vacuole, it seems probable that their membranes fuse with that of the vacuole and so there will be enough
membrane made available for the vacuole to assume a shape other than spherical. It may be that a vacuole with a large surface area makes use of it to facilitate passage of lysosomal enzyme protein in and ferritin and other materials out. Why they should stay this shape after ID digestion is interesting, but it is possible that the vacuoles may be terminal and filled with toxic waste products.

In ID vacuoles it is common to see a 'halo' between the membrane and contents. Daems et al. (1969) describe this as a feature of lysosomes but do not suggest a reason for it. As it is so constant a feature, it could be that there is some electron-lucent coat within the membrane which prevents contents from lying close to it. In vacuoles with only a little ID or ferritin it is frequently aligned in a regular fashion inside this halo.

One of the intriguing questions in this study is the location of the site of apoferritin and ferritin synthesis. Undoubtedly in an organ which normally handles iron there will be some apoferritin in the cells. Richter (1959) mentions
this, but it has also been proved by Fineberg & Greenberg (1955a, b) and Yoshino et al. (1968) that de novo synthesis of apoferritin is accelerated by the administration of iron. Iron in the form of ID is in the vacuoles so information must pass from the site of ID to the site of apoferritin synthesis. Yoshino et al. show that the uptake of $^{14}$C orotic acid into nuclear RNA is increased by iron administration and so favour the view that apoferritin synthesis is stimulated by a rise in production of m-RNA. All these authors agree that iron does not increase ferritin synthesis simply by converting iron-poor into iron-rich ferritin.

What happens after the m-RNA stimulation remains obscure. Fineberg & Greenberg (1955a) show that in guinea pig even after iron stimulation the apoferritin synthesis only represents 3% of total protein synthesis, so it is quite likely that there would be no noticeable changes in RER and Golgi membranes. However, if all the apoferritin is made on RER it must be transported to the ID vacuoles because at least some ferritin synthesis takes place there
judging by the early concentration of ferritin observed in the vacuoles. If apoferritin is transported via Golgi vesicles, as lysosomal enzymes appear to be, then it is conceivable that the two proteins could be transported in the same manner to the ID vacuoles.

There is the problem that the lysosomal enzymes might attack the apoferritin inside the vacuoles. Observations show the supposed enzyme in local patches in the vacuole, and also ID is broken down in patches, and ferritin appears in patches, so perhaps the lysosomal enzymes do not diffuse to any great extent. Enzymes could enter the vacuole in Golgi vesicles and supposing the pH and other conditions are optimum for those attacking dextran, the ID could be broken down and the apoferritin could either enter then or already be in situ. Presumably the various lysosomal enzymes do not all work under the same conditions so where dextran is broken down apoferritin is not attacked. Combination of apoferritin and iron micelles could then take place.

Electron-lucent areas within ID vacuoles
could be the sites of apoferritin storage. These are seen in this study and have been reported by Richter (1957), Bessis & Breton-Gorius (1959) and Muir (1960). It is even possible that apoferritin may be synthesised within the vacuoles (Muir & Golberg, 1961) although this does seem unlikely in the light of current knowledge of protein synthesis.

Very small micelles of iron, sometimes in a string-of-beads formation, are seen occasionally and are also reported by Richter (1958). There are three possible explanations for their presence; either they are the breakdown products of FeOOH and are about to be attached to apoferritin as larger micelles, or they are partially filled cores of ferritin, or they are the breakdown products of ferritin iron. Since ferritin is naturally in the liver it may be degraded by the lysosomal enzyme digestion.

The possibility remains that ferritin could be made in the cytoplasm. In this study it seems unlikely as a low concentration of ferritin is seen in the Kupffer cell matrix. The only
other iron ever seen in the cytoplasmic matrix is ID which is observed very rarely and is interpreted as being an artefact of sectioning.

The ferritin concentration in the cytoplasmic matrix of parenchymal cells is at most times much higher than in the Kupffer cells. From the amount of ID available in the two types of cell it seems that ID must be converted to ferritin in the Kupffer cell which then passes into the parenchymal cell. If most ferritin, and therefore most apoferritin, is made in the Kupffer cells it could be that the removal of these iron storage proteins stimulates production of more apoferritin as suggested by Fineberg & Greenberg (1955b).

4.4.3 Kinetics

Clearance of ID from the blood in the dose concentration of 500 mg Fe/Kg takes between 24 and 48 hr. It is not known what the rate limiting factor is but, from the observations on uptake and the evidence presented by other authors interested in opsonins, it could be saturation of the opsonins. The atomic absorption spectrometry results show that 6%
of the total ID load/hr is removed in the first 4 hr, but thereafter the rate falls because 20% of the total load remains in the blood at 24 hr. It could be that opsonin supply cannot continue at the initial pace and so diminishes to control the ID uptake rate. It may also be that the number of lining cells in a suitable metabolic state limits the uptake, so more lining cells may be converted to a suitable state, and would explain why uptake increases after a slow start as shown by atomic absorption spectrometry, and then slows again as the colloid load decreases. Another factor may be the amount of energy available for the cell to use in the known energy dependent process of phagocytosis.

The digestion of ID by lysosomal enzymes is limited by the rate of protein synthesis of the ribosomes and efficiency of the Golgi areas in transporting the enzymes to the ID vacuoles. The indefinite rise in enzyme concentration is difficult to explain. It is conceivable that once a Kupffer cell has ingested a vast amount of material and its enzyme synthesis has been
raised to a very high pitch, it remains at that level throughout its life, as shown in rats 1 yr after ID injection. It remains an interesting point to debate as to whether this effect is due to a young lining cell ingesting an old cell and digesting it, thus keeping the lysosomal concentration in Kupffer cells high.

Why the lining cells ingest the ID to such an extent and the parenchymal cells contain so much ferritin is also intriguing. If, as has been postulated, the ferritin is made in the Kupffer cells and transferred to the parenchymal cells it may be that it is stored in them in preference to Kupffer cells perhaps because they have a longer life span, or it may be en route to some other storage site. Unless apoferritin is protein predominantly made in Kupffer cells, it seems curious that the ferritin synthesis should be there since parenchymal cells are so much richer in ribosomes.

4.5 Conclusion

In this study there is an absence of long-term change in lining cells and parenchymal cells apart from haemosiderin or ferritin storage.
No fibrosis, necrosis or signs of liver cell division are seen and the cells of the liver seem to have handled a huge load of colloid most successfully.


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APPENDIX I

The author wishes to acknowledge the assistance of Dr. D. Rutherford in the following calculation, for which she expresses her thanks.

Calculation of number of particles in FPL 2000/2001 solutions

Assumption: that FPL 2000 = 2001 in terms of particle size and that the EM profiles are prolate spheroids of B-FeOOH.

Calculation: Avogadro's Number ($N$), the number of individual atoms in a g atom = $6.023 \times 10^{23}$

\[ \therefore \text{1 atom of Fe weighs } \frac{55.85}{6.023 \times 10^{23}} \text{ g} \]

\[ = 9.272 \times 10^{-23} \text{ g} \]

FPL 2000/2001 contains 20% Fe (W/V) i.e. 0.200 g/ml

\[ \therefore \text{No. atoms Fe/ml in 2000 solution} = \frac{0.200}{9.272 \times 10^{-23}} \]

\[ = 2.157 \times 10^{21} \]

(Volume of FeOOH core is 678.7 nm$^3$, and 0.323 nm$^3$ is the volume of 1 unit of FeOOH,
but only polymers of FeOOH exist, containing 8 atoms of Fe.)* Note 1

Then, number of atoms Fe/particle

\[
\text{Number of atoms Fe/particle} = \frac{8 \times 678.7}{0.332} = 1.634 \times 10^4
\]

Number of particles/ml solution

\[
\text{Number of particles/ml solution} = \frac{2.157 \times 10^{21}}{1.634 \times 10^4} = 1.323 \times 10^{17}
\]

* Note 1: Minshull, R. Personal communication to Dr. Rutherford.
FIGURE 1. Diagrammatic representation of a hepatic sinusoid.

L - lumen of sinusoid
K - Kupffer cell
S - sinusoidal lining cell
P - parenchymal cell
Pe - perisinusoidal cell
SD - space of Disse
N - nucleus
M - mitochondrion
RER - rough endoplasmic reticulum
G - glycogen
GA - Golgi area
R - reticulin
V - vesicles
F - lipid droplet
Ly - lysosome
BC - bile canaliculus
FIGURE 2. Perfusion apparatus.

A pre-fixation wash is in the left syringe, and fixative in the right. The pressure is adjusted by altering the height of the central limb and the solution enters the portal vein through the front cannula.
FIGURE 3.  a) H & E  b) PAS
c) Perl's Prussian Blue  d) Toluidine Blue
Reactions

Fixation:  a, b and c fixed in HgCl₂ and formaldehyde; d fixed in 1% OsO₄ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

7 wk:  control  a,b,c  x 430
d  x 1,200
FIGURE 4. Acid phosphatase shown by

a) Gomori method. The dark brown reaction product indicates acid phosphatase.

b) Barka method. The red reaction product indicates acid phosphatase.

The reaction product in both is predominantly in parenchymal cells. In the Gomori method a few Kupffer cells have brown reaction product, but this is not seen with the Barka reaction except very occasionally.

7 wk: control x 480
FIGURE 5. Montage of a sinusoidal lining cell.

Fixation:  1% OsO$_4$ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain:  Uranyl acetate II, Reynolds' lead citrate

7 wk:  control  x 34,000
FIGURE 6. Sinusoid showing the lining cells, space of Disse, containing microvilli from surrounding parenchymal cells, and a perisinusoidal cell containing a lipid droplet. Fenestrations are seen in one lining cell, and in another there are coated vesicles under the luminal surface.

Fixation: 1% OsO$_4$ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds lead citrate

7 wk: control x 19,500
FIGURE 7. Sinusoidal lining cells of different shapes, with coated vesicles on both luminal and abluminal surfaces. One of the cells contains prominent ER, alternating between rough and smooth, and the same cell has various inclusions, probably secondary lysosomes. A portion of a perisinusoidal cell containing a lipid droplet is seen.

Fixation: 1% OsO₄ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds' lead citrate

7 wk: control x 30,000
FIGURE 8. Lining cell bridging a sinusoid.

Circular sphaeridies (→) are seen in the nucleus and several areas of Golgi membranes lie within a concavity of the nucleus. Fenestrations in the thin lining cells are seen.

Fixation: 1% \( \text{OsO}_4 \) in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds' lead citrate

7 wk: control x 17,000
FIGURE 9. A large rounded lining cell with several areas of Golgi membranes. Numerous circular vacuoles filled with heterogeneous granular material are seen, and within the nucleus there is a nucleolus. Only a small area of the abluminal surface is contributing to the lining of the sinusoid, the remainder overlies other lining cells, and bridges the sinusoid. Flocculent material lies close to the cell membrane in many places.

Fixation: 1% OsO₄ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds' lead citrate

7 wk: control x 24,500
FIGURE 10. Portion of a flat sinusoidal lining cell with a smooth surface. Coated vesicles in various stages of formation are seen on both surfaces.

Fixation: Wash with 0.15M sodium cacodylate, pH 7.4, then fixation 1% OsO₄ in 0.1M sodium cacodylate, pH 7.4 at 37°C.

Stain: Lead citrate, uranyl acetate I

34 wk: control x 40,000
FIGURE 11. Lining cell with many coated vesicles on the luminal surface. The luminal membrane is less sharply defined than the abluminal surface. For the greater part of its surface this cell is overlying other lining cells.

Fixation: 1% OsO₄ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds lead citrate

7 wk: control x 26,000
FIGURE 12. Portion of a lining cell showing an area of micropinocytosis vermiformis (MV). Several vacuoles have the appearance of distended micropinocytosis vermiformis. Both surfaces have coated vesicles. This cell contains several areas of Golgi membranes and many vacuoles of various sizes with heterogeneous contents. Close to the area of micropinocytosis vermiformis several thrombocytes (T) are seen lying in the sinusoid.

Fixation: 1% OsO$_4$ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds' lead citrate

7 wk: control x 16,000
FIGURE 13. The same cell as in Fig 12 showing more clearly the profiles of micropinocytosis vermiformis. The distended, vacuolar profiles contain flocculent osmiophilic material similar to that in the middle of the worm-like profiles. A large part of the cell surface has a similar coating.

Fixation: 1% OsO₄ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds’ lead citrate

7 wk: control x 32,500
FIGURE 14. A lining cell with a smooth abluminal surface and a tortuous luminal surface. A few profiles of micropinocytosis vermiformis are seen luminally. The cell contains many vacuoles and two residual bodies enclosing myelin figures. Flocculent material is seen close to the luminal surface.

Fixation: 1% OsO₄ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds’ lead citrate

59 wk: control x 19,500
FIGURE 15. A lining cell with many cytoplasmic projections from its luminal surface, and an erythrocyte in close apposition to it. The cell contains many phagocytic vacuoles, the largest of which has dense contents similar in appearance to the erythrocyte.

Fixation: Wash with 0.15M sodium cacodylate, pH 7.4, then fixation 1% OsO₄ in 0.1M sodium cacodylate, pH 7.4 at 37°C.

Stain: Lead citrate, uranyl acetate I

21 wk: control x 13,000
FIGURE 16. A rounded lining cell bridging a sinusoid. The surface is very tortuous, with many cytoplasmic projections. In the cell Golgi areas are prominent as well as RER, SER and phagocytic vacuoles. A centriole (→) is located near the centre of the cell. Reticulin fibres are in the space of Disse.

Fixation: 1% OsO$_4$ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds' lead citrate

7 wk: control x 13,000
FIGURE 17. A rounded lining cell with a tortuous surface containing an abundance of free ribosomes and three large vacuoles containing dense granular material and in one case membranes. The nucleus is cut tangentially and shows nuclear pores.

Fixation: 1% OsO$_4$ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds' lead citrate

14 wk: control x 26,000
FIGURE 18. The same cell as Fig 17 showing more clearly the nuclear pores (→) and whorls of ribosomes. The three large vacuoles have a halo between the granules and their membrane. Several smaller vacuoles have low concentrations of ferritin.

Fixation: 1% OsO₄ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds lead citrate

14 wk: control x 55,000
FIGURE 19. A lining cell completely filling a sinusoid. It has many vacuoles, some containing dense granular material and others also containing the remains of organelles.

Fixation: 1% OsO₄ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds’ lead citrate

59 wk: control x 13,000
FIGURE 20. A lining cell with several cytoplasmic projections. Its cytoplasmic matrix is noticeably less dense than that of adjacent hepatic parenchymal cells. Transition of endoplasmic reticulum from RER to SER is seen in this cell.

Fixation: 1% OsO₄ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds' lead citrate

7 wk: control x 32,500
FIGURE 21. A lining cell showing a tortuous surface, containing many large vacuoles in which there is material of both moderate and extreme electron-density. Some of the vacuoles contain a few profiles of ferritin, and in two vacuoles remnants of membranes are included.

Fixation: 1% OsO₄ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds’ lead citrate

59 wk: control x 21,000
FIGURE 22. A lining cell similar to that in Fig 21 except its outline is smooth. Several vacuoles appear to have coalesced to form larger ones. There is less of the moderately dense material and more extremely dense in this cell.

Fixation: 1% CsO₄ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds' lead citrate

59 wk: control x 16,000
FIGURE 23  a) ID diluted in water and viewed on a carbon-coated grid. x 300,000

b) The same ID at higher magnification showing lattice lines in some of the crystals. These lines probably represent parallel planes within the crystal which are only seen in particular orientations. x 500,000
FIGURE 24. A graph to show the rate of removal of ID from the blood. Each point represents the mean of the 6 results obtained, 3 from each experiment.
FIGURE 25. Perls' Prussian Blue reaction on tissue at different times after injection of ID 400 mg Fe/Kg

a) After 10 min. There is no demonstrable iron within any liver cells.

b) After 1 d. Over Kupffer cells there are large dense blue masses, and punctate blue widely distributed within parenchymal cells.

c) After 16 d. Kupffer cells have large dense blue masses over them, but punctate blue has been replaced by a much more diffuse blue.

d) 1 yr after 500 mg Fe/Kg. A few Kupffer cells show dense, but smaller blue aggregates of stain over them, and a great deal of diffuse blue is seen throughout the tissue.

x 480
FIGURE 26. Acid phosphatase as shown by the Gomori method after 400 mg Fe/Kg.

a) After 2 hr. An increase in reaction product over Kupffer cells is seen compared with control tissue. That in parenchymal cells remains unchanged.

b) After 16 d. Many Kupffer cells show increased quantities of reaction product.

c) After 52 wk. Fewer cells show increase in reaction product, but the few that do have a large increase.
FIGURE 27. Tissue from the same animals as in Fig 26, showing acid phosphatase by the Barka method.

a) After 2 hr. An increase in reaction product over Kupffer cells is again demonstrated, while parenchymal cells appear as controls.

b) After 16 d. Many Kupffer cells show increased quantities of reaction product. Brown ID is seen in Kupffer cells mixed with the reaction product.

c) After 52 wk. Fewer cells show increase in the reaction product, but a few large masses of it remain.
FIGURE 28. A portion of a sinusoidal lining cell showing uptake of ID. It has a very tortuous outline, under which numerous coated vesicles lie, some containing a few particles of ID. Micropinocytosis vermiciformis is seen at the extremity of the cell and deep within the cytoplasm are several large vacuoles with an osmiophilic lining in which ID is seen; these bear a resemblance to micropinocytosis vermiciformis which has distended. ID is within several vacuoles of moderate electron-density, as well as in a secondary lysosome. It is in the sinusoid, lying close to the cell membrane in many places and some is in the space of Disse and smaller lining cells. A thrombocyte is in the area of great pinocytotic activity.

Fixation: 1% OsO₄ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds' lead citrate

7 wk: + 500 mg Fe/Kg x 14,000
FIGURE 29. A portion of a sinusoidal lining cell showing a tortuous outline toward the sinusoid. ID is in the sinusoid apparently bound together with flocculent material. It is entering the cell in one invagination and in several coated vesicles, some of which have coalesced. It is also in micropinocytosis vermiformis (left) which has formed on the abluminal surface and is in the process of distending, and the other already distended profiles lie deeper in the cell. The ID is also in secondary lysosomes. This cell shows very many SER profiles. Numerous small electron-dense vesicles are throughout the cell.

Fixation: 1% OsO₄ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds' lead citrate

7 wk: + 500 mg Fe/Kg 10 min
previously x 28,000
FIGURE 30. A portion of a smooth surfaced lining cell with ID in coated vesicles, secondary lysosomes and in distended ER (top right). Many small electron-dense vesicles are throughout the cell. Fine cytoplasmic filaments are present, and there is what appears to be a branching mitochondrion.

Fixation: Wash with 0.15M sodium cacodylate, pH 7.4 at 37°C, then fixation 1% OsO₄ in 0.1M sodium cacodylate, pH 7.4 at 37°C.

Stain: Lead citrate, uranyl acetate I

7 wk: + 400 mg Fe/Kg 10 min
previously x 60,000
FIGURE 31. Adjacent hepatic parenchymal cells showing their intercellular space into which ID has penetrated. Ferritin is seen in the two dense bodies, as well as some in the cytoplasm which is also rich in glycogen.

Fixation: Wash with 0.15M sodium cacodylate, pH 7.4 at 37°C, then fixation 1% OsO₄ in 0.1M sodium cacodylate, pH 7.4 at 37°C.

Stain: Lead citrate, uranyl acetate I

7 wk: + 400 mg Fe/Kg 10 min previously x 45,000
FIGURE 32. Portion of a lining cell showing ID in micropinocytosis vermiformis where it is concentrated mainly along the central osmiophilic material. Some ID is lying close to a coated vesicle (top right).

Fixation: Wash with 0.15M sodium cacodylate, pH 7.4 at 37°C, then fixation 1% OsO₄ in 0.1M sodium cacodylate, pH 7.4 at 37°C.

Stain: Lead citrate, uranyl acetate

7 wk: + 400 mg Fe/Kg 20 min
previously x 80,000
FIGURE 33. **IL is seen in coated vesicles, lying close to the cell surface and also in secondary lysosomes which have arisen from autophagic vacuoles. Some coated vesicles lie near the largest of these, suggesting a possible transport mechanism. Also within the lysosome are round areas of moderate electron-density.**

**Fixation:** Wash with 0.15 M sodium cacodylate, pH 7.4 at 37°C, then fixation 1% OsO₄ in 0.1M sodium cacodylate, pH 7.4 at 37°C.

**Stain:** Lead citrate, uranyl acetate I

7 wk: + 400 mg Fe/Kg 20 min previously x 72,000
FIGURE 34. A large secondary lysosome which has apparently formed as a result of engulfment of several autophagic vacuoles (right). It contains ID and various round areas of moderate electron-density. Below this is another lysosome, containing ID, some of which in the centre is bound within another membrane suggesting engulfment. Again, dense areas are found. The relationship of these two vacuoles to each other suggests they may be about to coalesce.

Fixation: Wash with 0.15M sodium cacodylate, pH 7.4 at 37°C, then fixation 1% OsO₄ in 0.1M sodium cacodylate, pH 7.4 at 37°C.

Stain: Lead citrate, uranyl acetate

7 wk: + 400 mg Fe/Kg 20 min previously x 75,000
FIGURE 35. A portion of a sinusoidal lining cell showing ID entering the cell in various invaginations (see Fig 36). Within the cell are many vacuoles of ID, one dumbbell-shaped, suggesting coalescence. In each is moderately dense material.

Fixation: Wash with 0.15M sodium cacodylate, pH 7.4 at 37°C, then fixation 1% OsO₄ in 0.1M sodium cacodylate, pH 7.4 at 37°C.

Stain: Lead citrate, uranyl acetate I

7 wk: + 400 mg Fe/Kg 20 min previously x 25,000
FIGURE 36. The same cell as in Fig 35, showing more clearly the invaginations containing ID. In the vacuoles the electron-dense material is seen, and surrounding these are small vesicles of the same density. Filaments are present in the cytoplasm.

Fixation: Wash with 0.15M sodium cacodylate, pH 7.4 at 37°C, then fixation 1% OsO4 in 0.1M sodium cacodylate, pH 7.4 at 37°C.

Stain: Lead citrate, uranyl acetate

7 wk: + 400 mg Fe/Kg 20 min previously x 50,000
FIGURE 37. At the top of the micrograph is a portion of a sinusoidal lining cell containing ID in two vacuoles with what appears to be a bridge between them. This could be a tubular structure, appearing thus due to the plane of section. Electron-dense material is mixed with ID. In the lower part, ID is in the space of Disse and in several vacuoles in a hepatic parenchymal cell.

Fixation: Wash with 0.15M sodium cacodylate, pH 7.4 at 37°C, then fixation 1% OsO₄ in 0.1M sodium cacodylate, pH 7.4 at 37°C.

Stain: Lead citrate, uranyl acetate

7 wk: + 400 mg Fe/Kg 20 min previously x 87,000
FIGURE 38. ID in vacuoles within a sinusoidal lining cell. The large, round vacuole has two vesicles of moderate electron-density lying close to it. One of the other vacuoles has a long thin extension containing only a few crystals of ID. Small moderately dense vesicles are in the vicinity of the vacuoles.

Fixation: Wash with 0.15M sodium cacodylate, pH 7.4 at 37°C, fixation 1% OsO₄ in 0.1M sodium cacodylate, pH 7.4 at 37°C.

Stain: Lead citrate, uranyl acetate

7 wk: +400 mg Fe/Kg 2 hr previously x 70,000
FIGURE 39. A hepatic parenchymal cell showing dense bodies containing ID and small vesicles containing ID close to the bile canaliculus.

Fixation: Wash with 0.15M sodium cacodylate, pH 7.4 at 37°C, fixation 1% OsO₄ in 0.1M sodium cacodylate, pH 7.4 at 37°C.

Stain: Lead citrate, uranyl acetate

7 wk: +400 mg Fe/Kg 2 hr previously x 38,000
FIGURE 40. A sinusoidal lining cell showing many vacuoles of ID also with electron-dense contents. Several of the vacuoles are dumbell-shaped. ID is present in invaginations and coated vesicles; there is also ID in vesicles in the hepatic parenchymal cell and the space of Disse.

Fixation: Wash with 0.15M sodium cacodylate, pH 7.4 at 37°C, fixation 1% OsO₄ in 0.1M sodium cacodylate, pH 7.4 at 37°C.

Stain: Lead citrate, uranyl acetate

7 wk: +400 mg Fe/Kg 3 hr previously x 20,000
FIGURE 41. Portion of a sinusoidal lining cell containing several vacuoles of ID. In most cases the ID is loosely packed, or mixed with electron-dense material. A Golgi area is seen with vesicles containing similar electron dense material close to it, and also scattered throughout the cytoplasm.

Fixation: Wash with 0.15M sodium cacodylate, pH 7.4 at 37°C, fixation 1% OsO₄ in 0.1M sodium cacodylate, pH 7.4 at 37°C.

Stain: Lead citrate, uranyl acetate

7 wk: +400 mg Fe/Kg 3 hr previously x 55,000
FIGURE 42. Adjacent hepatic parenchymal cells with a bile canalculus between them. Several secondary lysosomes are seen containing ID and some with ferritin.

Fixation: Wash with 0.1M sodium cacodylate, pH 7.4 at 37°C, fixation 1% OsO₄ in 0.1M sodium cacodylate, pH 7.4 at 37°C.

Stain: Lead citrate, uranyl acetate I

7 wk: +400 mg Fe/Kg 3 hr previously x 43,000
FIGURE 43. Portion of a sinusoidal lining cell showing a little ID in coated vesicles and, at the top, some in a vacuole. A centriole is seen on the luminal aspect of the nucleus, and coated vesicles on both surfaces. Some of those on the luminal aspect have ID in them.

Fixation: 1% OsO$_4$ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds' lead citrate

7 wk: +500 mg Fe/Kg 17 hr previously x 40,000
FIGURE 44. Another sinusoidal lining cell containing many vacuoles of ID. At this time many are of dumbell, or more tortuous shapes. The ID is tightly packed in most vacuoles and the electron-dense material seen at shorter periods after injection is either absent or obscured. Some ID remains outside the cell.

Fixation: 1% OsO₄ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds lead citrate

7 wk: +500 mg Fe/Kg 17 hr previously x 23,000
Another sinusoidal lining cell showing many vacuoles of ID. Many of these show areas of dense material mixed with ID, and also some electron-lucent areas. There is continued presence of ID in coated vesicles.

Fixation: 1% OsO₄ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds lead citrate

7 wk: +500 mg Fe/Kg 17 hr

previously x 25,000
FIGURE 46. Portion of a sinusoidal lining cell showing large densely packed vacuoles of ID. Most vacuoles are not circular in profile, but have a variety of shapes. They contain electron-dense material and electron-lucent areas in with the ID. In this section some ID is apparently free in the cytoplasm, which may be an artefact due to sectioning.

Fixation: Wash with 0.15M sodium cacodylate, pH 7.4 at 37°C, fixation 1% OsO₄ in 0.1M sodium cacodylate, pH 7.4 at 37°C.

Stain: Lead citrate, uranyl acetate I

7 wk: +400 mg Fe/Kg l d
previously x 90,000
FIGURE 47. Portion of a sinusoidal lining cell showing part of a vacuole containing rounded areas of ID and ferritin. Some electron-dense material lies within the vacuole and there is a siderosome just outside the vacuole.

Fixation: Wash with 0.15M sodium cacodylate, pH 7.4 at 37°C, fixation 1% OsO₄ in 0.1M sodium cacodylate, pH 7.4 at 37°C.

Stain: Lead citrate, uranyl acetate

7 wk: +400 mg Fe/Kg 2 d
previously x 80,000
FIGURE 48. Two adjacent hepatic parenchymal cells showing dense bodies in their peribiliary regions. They contain vacuoles of ferritin or haemosiderin and one vacuole contains ID which is also seen in the lumen of the bile canaliculus.

Fixation: Wash with 0.15M sodium cacodylate, pH 7.4 at 37°C, fixation 1% OsO₄ in 0.1M sodium cacodylate, pH 7.4 at 37°C.

Stain: Lead citrate, uranyl acetate

7 wk: +400 mg Fe/Kg 2 d previously x 19,000
FIGURE 49. A large tortuous vacuole containing a mixture of ID and ferritin, with the highest concentration of ID around the edges. The cytoplasm surrounding the vacuole contains much ferritin.

Fixation: 1% OsO$_4$ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds' lead citrate

7 wk: +400 mg Fe/Kg 4 d

previously x 75,000
FIGURE 50. Portion of a sinusoidal lining cell showing a small vacuole of ID and ferritin. Adjacent vacuoles contain only a small amount of flocculent material and ferritin. The cytoplasmic matrix of the hepatic parenchymal cell contains much more ferritin than that of the sinusoidal lining cell.

Fixation: Wash with 0.15M sodium cacodylate, pH 7.4 at 37°C, fixation 1% OsO₄ in 0.1M sodium cacodylate, pH 7.4 at 37°C.

Stain: Lead citrate, uranyl acetate I

8 wk: 400 mg Fe/Kg 8 d
previously x 50,000
FIGURE 51. Portion of a sinusoidal lining cell showing a large vacuole containing ID and ferritin. A large part of the vacuole is devoid of iron and is surrounded by a layer of ferritin under the membrane. A large amount of ID in this, and other vacuoles, appears unchanged. In some vacuoles particles of iron smaller than ferritin are seen lying close to one another like beads on a string (→).

Fixation: Wash with 0.15M sodium cacodylate, pH 7.4 at 37°C, fixation 1% OsO₄ in 0.1M sodium cacodylate, pH 7.4 at 37°C.

Stain: Lead citrate, uranyl acetate

9 wk: +400 mg Fe/Kg 12 d
previously x 62,000
FIGURE 52. Portion of a sinusoidal lining cell containing vacuoles of ID, ID and ferritin mixed, and ferritin alone. A mitochondrion with ferritin and ID overlying it appears thus due to a tangential section being taken through an adjacent iron-containing vacuole.

Fixation: Wash with 0.15M sodium cacodylate, pH 7.4 at 37°C, fixation 1% OsO₄ in 0.1M sodium cacodylate, pH 7.4 at 37°C.

Stain: Lead citrate, uranyl acetate

9 wk: +400 mg Fe/Kg 12 d
previously x 60,000
FIGURE 53. A vacuole from a sinusoidal lining cell with ID, ferritin and smaller iron particles (→). It also contains some dense amorphous material.

Fixation: Wash with 0.15M sodium cacodylate, pH 7.4 at 37°C, fixation 1% OsO₄ in 0.1M sodium cacodylate, pH 7.4 at 37°C.

Stain: Lead citrate, uranyl acetate I

9 wk: +400 mg Fe/Kg 16 d previously x 160,000
FIGURE 54. Vacuoles containing ID and ferritin and smaller iron particles in a sinusoidal lining cell. In places large amounts of ID appear intact. There is much ferritin in the cytoplasmic matrix. Vesicles containing dense material are in the cytoplasm near some vacuoles.

Fixation: Wash with 0.15M sodium cacodylate, pH 7.4 at 37°C, fixation 1% OsO₄ in 0.1M sodium cacodylate, pH 7.4 at 37°C.

Stain: Lead citrate, uranyl acetate I

10 wk: +400 mg Fe/Kg 22 d previously x 50,000
FIGURE 55. A large tortuous vacuole containing ID and ferritin as well as remains of organelles inside a sinusoidal lining cell. Smaller iron particles are also present. A vesicle of electron-dense material lies near the vacuole. Ferritin lies in a rim under the vacuole membrane, leaving a well-defined halo.

Fixation: Wash with 0.1M sodium cacodylate, pH 7.4 at 37°C, fixation 1% OsO₄ in 0.1M sodium cacodylate, pH 7.4 at 37°C.

Stain: Lead citrate, uranyl acetate

14 wk: +500 mg Fe/Kg 7 wk

previously x 46,000
FIGURE 56. A large vacuole of ID, ferritin and remains of organelles in a sinusoidal lining cell. In smaller vacuoles of ferritin a distinct halo is seen under the membrane. There are many vesicles of electron-dense material in the vicinity of the vacuoles.

Fixation: Wash with 0.15M sodium cacodylate, pH 7.4 at 37°C, fixation 1% OsO₄ in 0.1M sodium cacodylate, pH 7.4 at 37°C.

Stain: Lead citrate, uranyl acetate I

14 wk: +60 mg Fe/Kg 7 wk previously x 53,000
FIGURE 57. Portion of a sinusoidal lining cell showing large vacuoles containing dense flocculent material which is closely associated with ferritin and smaller particles of iron.

Fixation: Wash with 0.15M sodium cacodylate, pH 7.4 at 37°C, fixation 1% OsO₄ in 0.1M sodium cacodylate, pH 7.4 at 37°C

Stain: Lead citrate, uranyl acetate

14 wk: +20 mg Fe/Kg 7 wk
previously x 67,000
FIGURE 58. Portion of a sinusoidal lining cell containing many vacuoles of ferritin, or haemosiderin, and a few of ID and ferritin. The halo under the membrane is very pronounced in some. Surrounding the vacuoles there is much ferritin in the cytoplasm. Vesicles of electron-dense material lie in the vicinity of the vacuoles. Many profiles of ER showing transitions from rough to smooth are seen.

Fixation: 1% OsO₄ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds lead citrate

21 wk: +500 mg Fe/Kg 14 wk previously x 40,000
FIGURE 59. A sinusoidal lining cell with a tortuous luminal membrane. It contains a vacuole of haemosiderin with a distinct halo. There is much ferritin in the cytoplasmic matrix.

Fixation: 1% OsO₄ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds lead citrate

21 wk: +500 mg Fe/Kg 14 wk
previously x 45,000
FIGURE 60. A sinusoidal lining cell showing micropinocytosis vermiformis in formation at the bottom of the micrograph, on the abluminal surface, and throughout the cell. Ferritin is contained in some small vacuoles. An erythrocyte is lying in close proximity to the cell which has a cytoplasmic extension beside it.

Fixation: 1% OsO₄ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds' lead citrate

34 wk: +20 mg Fe/Kg 27 wk previously x 25,000
FIGURE 61. Portion of a vacuole in a sinusoidal lining cell showing a few crystals of ID remaining and some ferritin mixed with it.

Fixation: 1% OsO₄ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds lead citrate

34 wk: + 20 mg Fe/Kg 27 wk previously x 110,000
FIGURE 62. A sinusoidal lining cell showing many vacuoles of haemosiderin, some having a tortuous shape.

Fixation: 1% OsO$_4$ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds' lead citrate

59 wk: + 500 mg Fe/Kg 1 yr previously x 28,000
FIGURE 63. The same cell as Fig 62 showing the haemosiderin under a halo within the membrane.

Fixation: $1\% \text{OsO}_4$ in $0.17\text{M}$ sodium cacodylate, pH 7.4 at $0^\circ\text{C}$.

Stain: Uranyl acetate II, Reynolds' lead citrate

59 wk: + 500 mg Fe/Kg
1 yr previously $\times 56,000$
FIGURE 64. A sinusoidal lining cell showing many vacuoles of haemosiderin or ferritin. There is much cytoplasmic ferritin throughout the tissue.

Fixation: 1% OsO₄ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds’ lead citrate

59 wk: +500 mg Fe/Kg 1 yr
previously x 24,000
FIGURE 65. A sinusoidal lining cell showing a large vacuole of haemosiderin surrounded by smaller ones of ferritin. There is much cytoplasmic ferritin throughout the tissue.

Fixation: 1% OsO₄ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds' lead citrate

59 wk: +500 mg Fe/Kg 1 yr
previously x 21,000
FIGURE 66. Many vacuoles of haemosiderin within a sinusoidal lining cell.

Fixation: 1% OsO$_4$ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds' lead citrate

59 wk: +500 mg Fe/Kg 1 yr
previously x 53,000
FIGURE 67. Vacuoles of haemosiderin or ferritin within a sinusoidal lining cell. The large central vacuole shows iron around the edge under the halo.

Fixation: 1% OsO₄ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds' lead citrate

59 wk: +500 mg Fe/Kg 1 yr
previously x 30,000
FIGURE 68. A sinusoid showing the lining cells, one of which has many vacuoles of haemosiderin or ferritin. This cell has prominent Golgi areas.

Fixation: 1% OsO$_4$ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds lead citrate

59 wk: +500 mg Fe/Kg 1 yr
previously x 15,000
FIGURE 69. A sinusoidal lining cell showing haemosiderin or ferritin aggregates. This cell has prominent Golgi areas. A large perisinusoidal cell storing fat is seen at the top of the micrograph. Lying close to the lining cell is an erythrocyte.

Fixation: 1% OsO₄ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds' lead citrate

59 wk: +500 mg Fe/Kg 1 yr previously x 19,000
FIGURE 70. Hepatic parenchymal cells showing vacuoles of haemosiderin or ferritin near a bile canaliculus. There is much ferritin in the cytoplasmic matrix.

Fixation: 1% OsO$_4$ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds' lead citrate

59 wk: +500 mg Fe/Kg 1 yr
previously x 30,000
FIGURE 71. A sinusoidal lining cell containing large vacuoles with flocculent electron-dense contents. Ferritin is seen in close association with the flocculence. Much SER in the form of tubules is seen at the bottom of the micrograph.

Fixation: 1% OsO₄ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds lead citrate

59 wk: +60 mg Fe/Kg 1 yr

previously x 20,000
FIGURE 72. Portion of a sinusoidal lining cell in which many secondary lysosomes are seen. Some contain myelin figures and remains of organelles, and some have flocculent contents of varying densities. Micropinocytosis vermiformis is forming on the luminal aspect of the cell and in one place it is distending.

Fixation: 1% OsO₄ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds lead citrate

59 wk: +20 mg Fe/Kg 1 yr
previously x 22,000
FIGURE 73. A sinusoidal lining cell showing various secondary lysosomes containing degraded organelles and in one, a myelin figure. A knob-like portion of another cell is in close apposition to this lining cell, at the top of the micrograph. At the bottom right there is a junction between it and a third cell.

Fixation: 1% OsO$_4$ in 0.17M sodium cacodylate, pH 7.4 at 0°C.

Stain: Uranyl acetate II, Reynolds lead citrate

59 wk: +20 mg Fe/Kg 1 yr
previously x 20,000
FIGURE 74. The same cell as in Fig 73, serially cut. Vacuoles can be traced, with more being seen in this plane of section. At the bottom the junction between this and another cell is seen.

Fixation: $1\% \text{OsO}_4$ in $0.17M$ sodium cacodylate, pH 7.4 at $0^\circ C$.

Stain: Uranyl acetate II, Reynolds' lead citrate

59 wk: $+20 \text{ mg Fe/Kg 1 yr}$

previously $x 20,000$
FIGURE 75. Hepatic parenchymal cells showing black deposit where acid phosphatase is located. This is mainly in the peribiliary area. Much ferritin is seen throughout these cells.

Fixation: 10% formaldehyde in 0.1M sodium cacodylate containing 1% CaCl$_2$. H$_2$O at pH 7.4 at 4°C for 6-7 hr.

Unstained

9 wk: +500 mg Fe/Kg 14 d
previously x 25,000
FIGURE 76. Hepatic parenchymal cells showing black deposit where acid phosphatase is located. This is in the peribiliary region. There is much ferritin throughout these cells.

Fixation: 10% formaldehyde in 0.1M sodium cacodylate containing 1% CaCl₂, H₂O, pH 7.4 at 4°C for 6-7 hr.

Unstained

9 wk: +500 mg Fe/Kg 14 d
previously x 32,000
FIGURE 77. Hepatic parenchymal cells showing black deposit where acid phosphatase is located. This is in the peribiliary region. There is much ferritin throughout these cells, some in vacuoles with black deposit.

Fixation: 10% formaldehyde in 0.1M sodium cacodylate containing 1% CaCl₂, H₂O, pH 7.4 at 4°C for 6-7 hr.

Unstained

9 wk: +500 mg Fe/Kg 14 d
previously x 45,000
FIGURE 78. Portion of a sinusoidal lining cell showing vacuoles of ID and ferritin. Because of their density it is impossible to tell if they contain acid phosphatase reaction product.

Fixation: 10% formaldehyde in 0.1M sodium cacodylate containing 1% CaCl₂. H₂O, pH 7.4 at 4°C for 6-7 hr.

Unstained

9 wk: +500 mg Fe/Kg 14 d
previously x 97,000
FIGURE 79. A diagrammatic representation of the uptake of ID and its fate at 1 yr after injection.
1 yr 500 mg Fe/kg