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FERRITIN FORMATION BY SYNOVIAL CELLS EXPOSED TO HAEMOGLOBIN *IN VITRO**

BY

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Recent electron microscope studies of synovium from patients with rheumatoid arthritis have shown that ferritin molecules are commonly found in the macrophage or type A lining cell (Muirden, 1966). Further studies have confirmed that deposition of iron is both common and extensive in rheumatoid synovial tissue.

Ferritin and haemosiderin, which are related in structure and function, represent the storage forms of iron in the body (Bothwell and Finch, 1962). Ferritin consists of ferric hydroxide-phosphate micelles specifically combined with the protein apoferritin. It is found particularly in the reticular cells of the bone marrow, liver, and spleen. The iron in the molecule comes either from plasma transferrin or from the breakdown of erythrocytes within reticular cells (Bessis and Breton-Gorius, 1962). Apoferritin is usually absent from cells but can be synthesized rapidly by a number of tissues on exposure to iron (Gabrio and Salomon, 1950).

Synthesis of ferritin has also been demonstrated in a variety of tissues. Since ferritin is not found in blood except in acute liver disease, it is likely that the ferritin found in rheumatoid synovium is formed in the joint.

There are two possible sources for the ferric moiety of synovial ferritin. It could arise from plasma transferrin, but this seems unlikely because extensive iron deposits occur in rheumatoid synovium despite an abnormally low level of serum iron (Muirden, 1967). However, it is possible that inflamed synovium may have an increased avidity for iron. Alternatively, the ferritin deposits might arise from haemoglobin, since red blood cells are common in rheumatoid synovial fluid and in the inflamed synovial membrane itself.

It is known that haemoglobin injected into animals through veins or peritoneum is transformed into ferritin in cells of the liver and kidney (Richter, 1957; Miller, 1960; Ericsson, 1964). Ferritin is formed in synovial cells after the intra-articular injection of organic iron salts (Ball, Chapman, and Muirden, 1964). The present study was designed to determine whether synovial cells could synthesize ferritin directly from haemoglobin without the mediation of other cells.

Our experimental model was to use synovial cells grown in culture and to add haemoglobin in very small concentration to the culture medium. The cells were fixed a short time later and subsequently examined with the electron microscope. Ferritin has a characteristic molecular structure which is identifiable on electron microscopy (Farrant, 1954). A similar system has been used by Richter (1961) to show ferritin formation in HeLa cells, but the stimulus was ferrous sulphate and not haemoglobin.

Material and Methods

Cultures of synovial cells from human knee joints were isolated and maintained by serial passage as described previously (Fraser and Catt, 1961; Fraser and McCall, 1965). Replicate cultures in maintenance growth medium were established in polystyrene Petri dishes and incubated at 37°C. in air with enough carbon dioxide to maintain *p*H within the physiological range. After 48 hours the growth medium was removed and replaced with fresh medium consisting of 33 to 40 per cent. human serum in Medium 199 (Morgan, Morton, and Parker, 1950). To prepare this serum, blood was taken from a fasting normal volunteer into two centrifuge tubes, and allowed to clot. One sample was used to prepare serum free of haemolysis for the control group of cultures. The other was partially haemolysed by vigorous agitation, or freezing and thawing, and then centrifuged at 3,000 rpm for 30 minutes. The haemoglobin content of this specimen was measured photometrically after reaction with acidified O-toluidine and hydrogen peroxide†, and

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the final haemoglobin iron concentration was calculated accordingly.

At the end of each experiment, the medium was removed and the cultures washed with Hanks's balanced salt solution. In four studies cells were detached by incubation and gentle agitation in 0·02 per cent. disodium versenate in Dulbecco-Vogt phosphate-buffered saline, then centrifuged and fixed in buffered 1 per cent. osmium tetroxide before embedding in Araldite. In the fifth study, the cells were fixed *in situ*, and removed with a plastic scraper. The tissue was stained either with 1 per cent. phosphotungstic acid during the dehydration stage or with a saturated solution of uranyl acetate after sectioning. Sections were examined in an Hitachi 11A electron microscope operating at 75 Kv.

Mucin clots were developed in the culture media with an equal volume of molar acetic acid. Further details of the cultures and their experimental treatment are shown in the Table.

Observations

One study was abandoned because of incomplete embedding. Another failed when the synovial cells suddenly lysed after serial passage of the culture through haemoglobin-containing media for 3 weeks. This followed immediately after application of a high concentration of haemoglobin.

In the five completed studies, exposure to haemoglobin was well tolerated. Light microscopy revealed no difference between individual cells in the control and treated groups, and in the longer experiments increased cell growth was apparent both by microscopy and by the bulk of the centrifuged pellets of cells, consistent with direct studies of cell growth-rates in the presence of low concentrations of haemoglobin (Pace and Bookhardt, 1960). Firm mucin clots were demonstrable in all the media before the experimental inoculations and in those of the control groups. In the test groups a brown precipitate appeared in the last three studies. After removal of this precipitate, the mucin clot was less bulky than in the controls and rather granular in character, suggesting that the precipitate may have bound some hyaluronic acid. However, the mucin clot was also deficient in those experiments where no spontaneous precipitation occurred with haemoglobin.

Electron Microscopic Observations

(*a*) *Control Cultures.*—The cells were loosely arranged with small quantities of finely granular material in the extra-cellular spaces (Fig. 1). The cell surfaces were mostly regular, with some finger-like processes (filopodia) and occasional pinocytotic vesicles. The nuclei contained one or more nucleoli and were bounded by a wavy membrane.

The cytoplasm varied from place to place in each cell. There were almost featureless areas containing a few particles resembling ribosomes, small vesicles, and vacuoles. In most areas, cytoplasmic organelles were more prominent. Mitochondria tended to be small. Scattered profiles of rough-surfaced endoplasmic reticulum occurred often near the nucleus, but in some cells it was more extensive and often parallel to the cell surface. The cisternae were narrow and contained material slightly denser than the cytoplasmic matrix. Scattered ribosomal particles were numerous. Intracellular filaments with a diameter of about 80 Å were occasionally seen. Golgi zones were frequent near the nucleus. There was also a variety of vesicles, vacuoles, and dense inclusions. The dense bodies were membrane-bounded and occasionally contained an internal structure suggesting that some might be derived from mitochondria. It was suspected that these structures contained lysosomal enzymes although histochemical proof was not sought. Following the terminology of Ericsson, Trump, and Weibel (1965), these membrane-bounded dense bodies will be referred to as "cytosomes" (Fig. 1). Close study of these

TABLE
PARTICULARS OF FIVE EXPERIMENTS

Study	Cell Strain	Age in Culture (days)	Number of Previous Passages	Haemoglobin Iron in Test Medium (μg./ml.)	Duration of Exposure Test Medium (hrs)
1	65–4	25	7	2·3	18
2	65–2	95	21	0·45	49
3	65–2*	34	6	4·8	48
4	65–2*	41	8	1·8	48
5	66–2	30	2	3·0 to 8·2	7 days (including subculture)

*. . . Substrain re-established from deep-freeze storage.

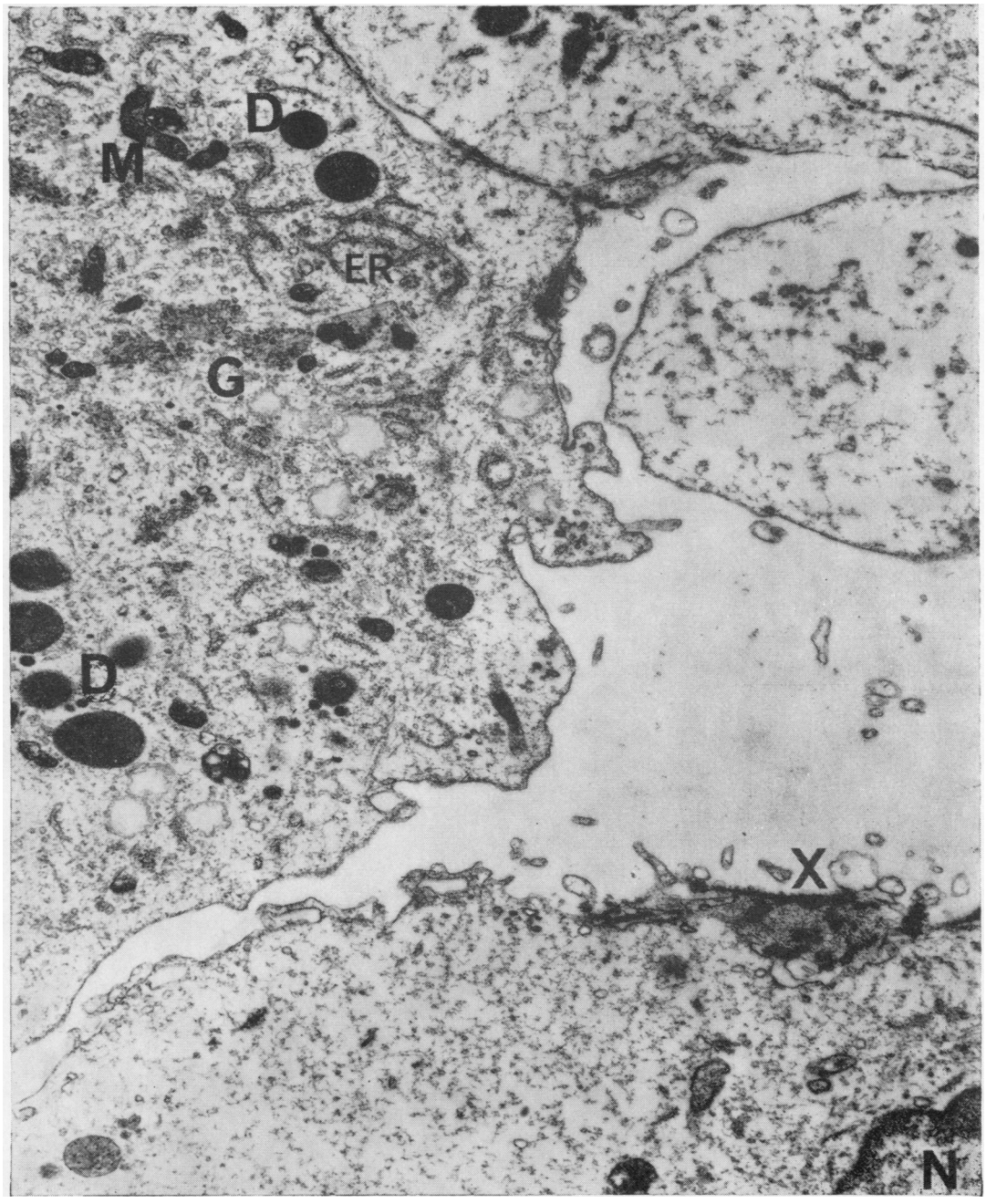


Fig. 1.—Control culture showing parts of four cells. The surfaces show small filopodia and a few pinocytotic vesicles. The cytoplasm is relatively featureless in places. One cells shows prominent dense bodies (D) or cytosomes and also small mitochondria (M), a Golgi zone (G), and some endoplasmic reticulum (ER). Higher magnification pictures did not show ferritin in the cytosomes. Some extracellular granular material appears at X. Study 3. ×14,800.

cytosomes in control cultures did not reveal the presence of ferritin-like granules.

(*b*) *Cultures with Haemoglobin.*—The structure of cells in these cultures was basically the same as that

just described. Filopodia were slightly more prominent, but degenerative changes, such as myelin figures, lipid droplets, and swollen mitochondria, were no more frequent. The most striking differences were found in study 3, where haemoglobin was precipitated in the culture medium, and appeared as irregular dense homogeneous masses in the extracellular spaces (Fig. 2). These were prominent

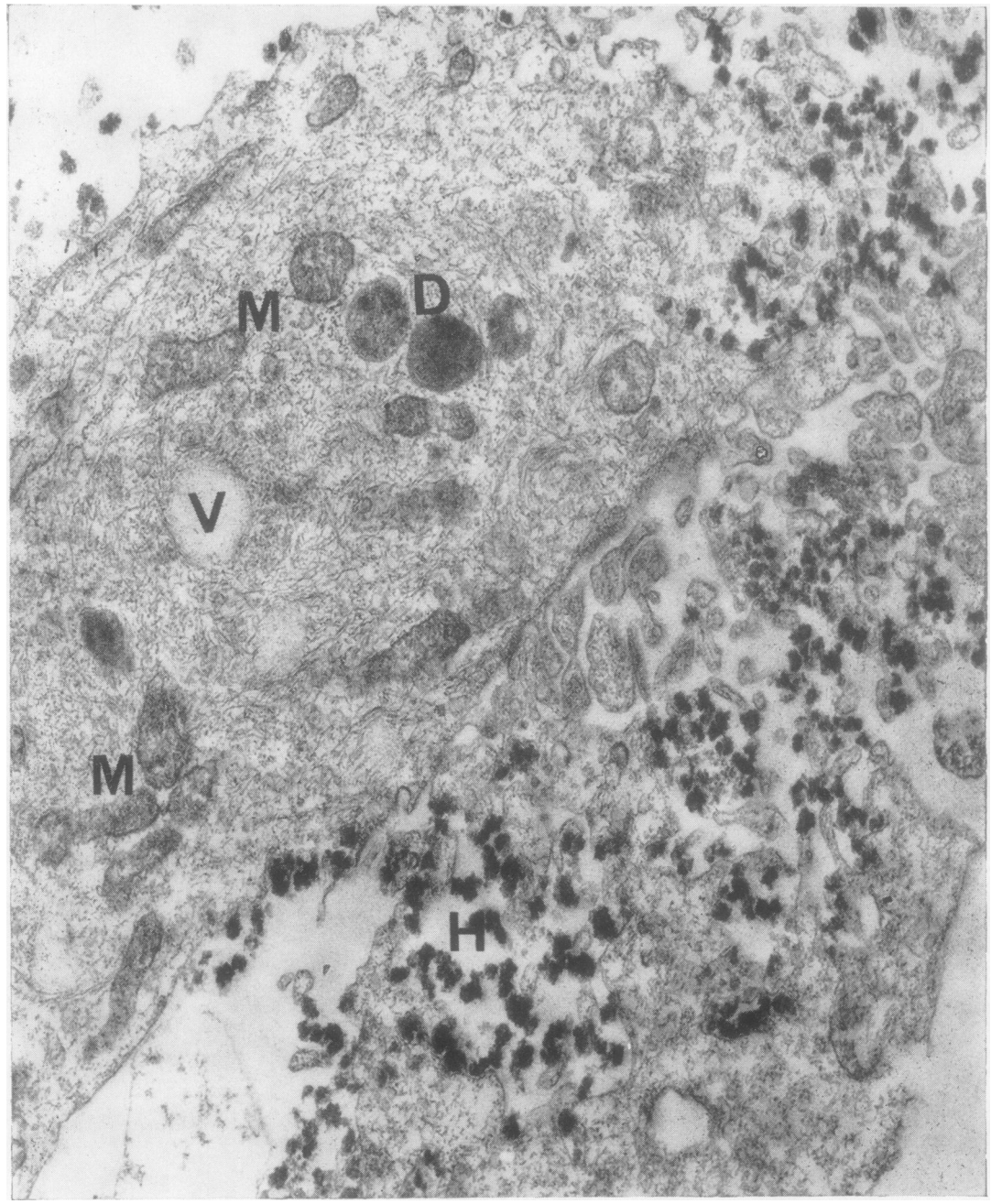


Fig. 2.—General view of two cells grown in media containing haemoglobin. The dense haemoglobin residues (H) are clearly seen and in places occupy surface vesicles and fill deep crypts in the cell membrane. Within the cell cytosomes are indicated (D) and also mitochondria (M) and a vacuole (V). Study 3. ×28,346.

within the folds of the cell surface and were seen in small surface pouches resembling pinocytotic vesicles (Fig. 3). Other views showed that the surface membrane had enclosed the haemoglobin particle within a complete vesicle. Large vacuoles also contained several haemoglobin fragments and their depth in the cell made it unlikely that they were cross-sections of crypts contiguous with the cell surface. Some sections revealed dense bodies in contact with phagocytic vacuoles (Fig. 3).

Fig. 4 (overleaf) shows a later stage in digestion and transformation of the haemoglobin residues. A membrane-bounded structure contains two particles similar to the haemoglobin masses seen outside the cell. The included particles were overall less dense but associated with small very dense granules. In other areas the haemoglobin residues were less evident but the granules more numerous. These granules were about 60 Å in diameter, and in high-resolution micrographs they showed the internal structure characteristic of the ferritin molecule (Fig. 5, overleaf).

In the experiments in which haemoglobin remained in solution, the early stages of the absorption of the haemoglobin fragments could not be shown. However, ferritin particles were clearly seen within dense bodies (Fig. 6, overleaf). These bodies had a diameter of up to 1·0 μ and were bounded by a single or occasionally double membrane. In a few, linear densities within the bodies suggested an origin from degenerate mitochondria. There was no constant relationship between the dense bodies containing

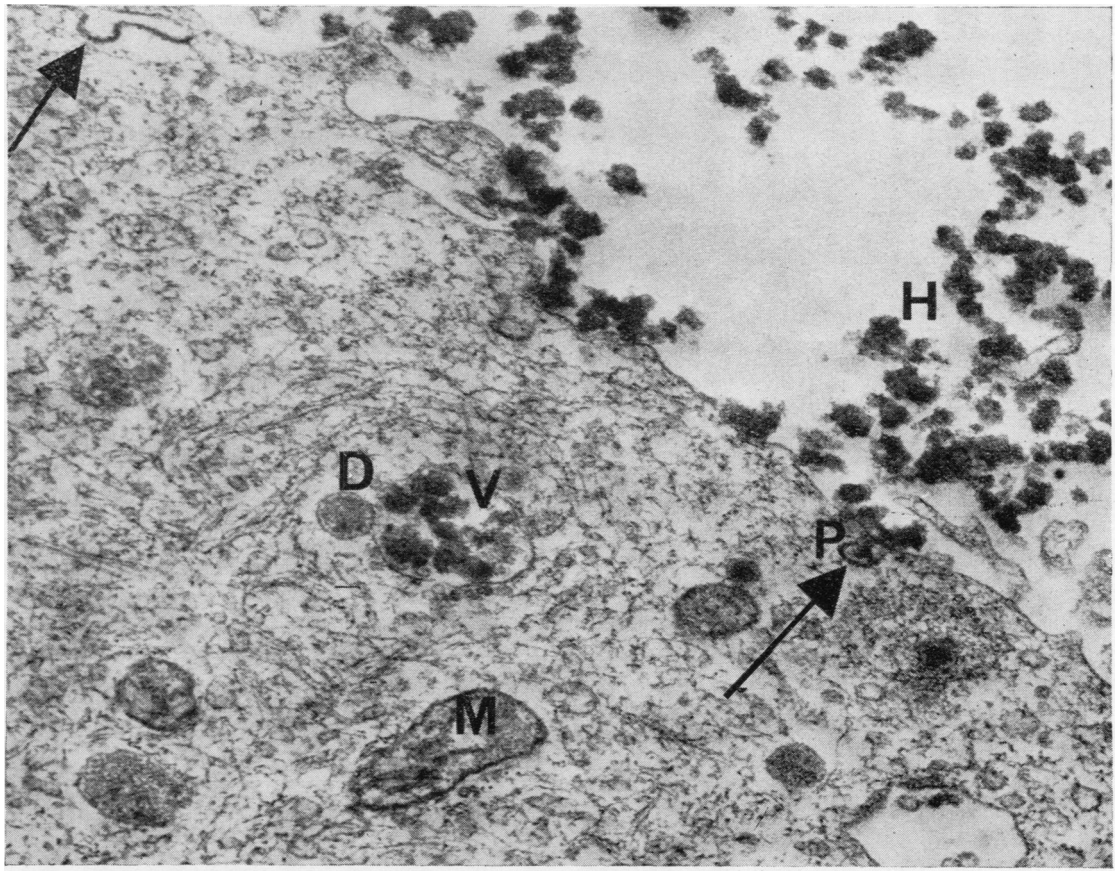


Fig. 3.—Haemoglobin residues (H) are seen outside a cell. Pinocytotic vesicles are indicated by arrows. That at P shows a particle within the lumen of the vesicle. A small cytosome (D) is shown in apposition to a vacuole containing many haemoglobin particles. A mitochondrion is indicated at M. Study 3. ×40,000

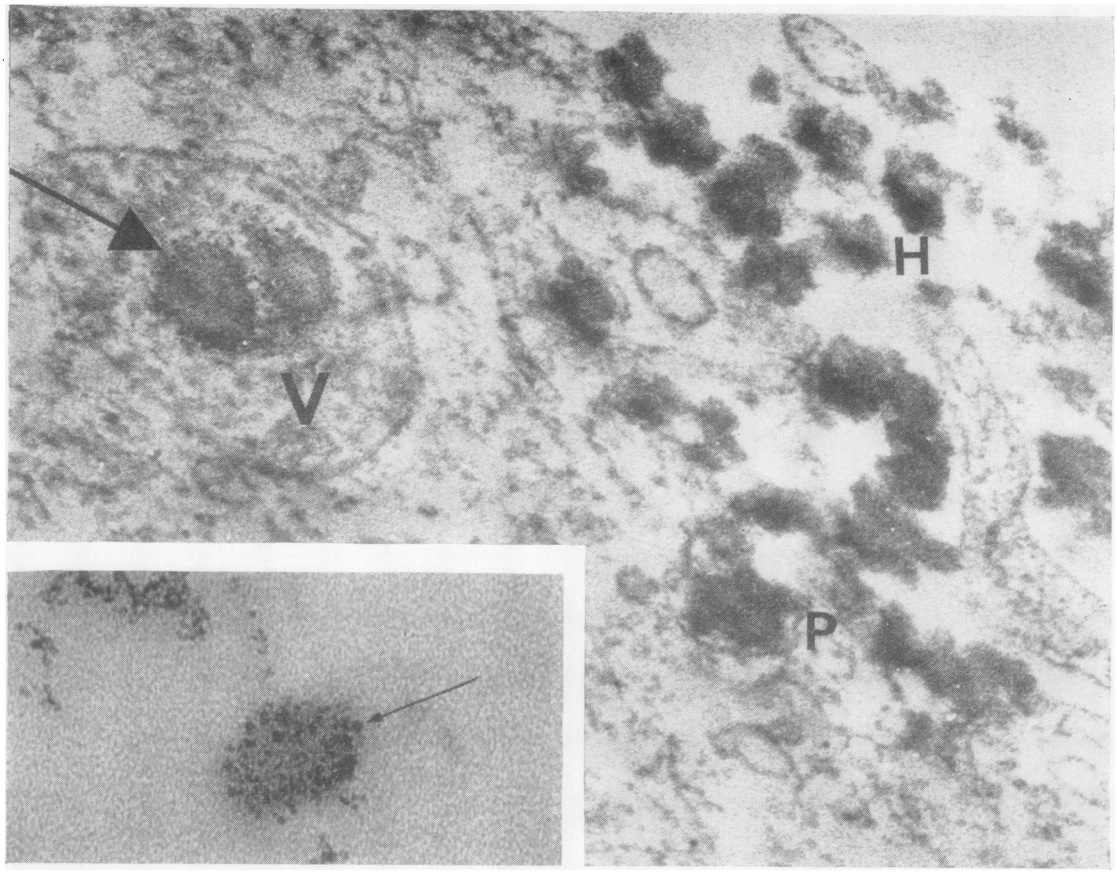


Fig. 4.—Haemoglobin residues (H) are seen external to a cell and in a pinocytotic vesicle (P). A large vacuole (V) shows two haemoglobin residues which are less dense than those external to the cell. Small dense granules appear around the haemoglobin residues (arrow). Study 3. ×90,000.

Fig. 5 (inset).—The typical tetrad structure of molecular ferritin is resolved (arrow). Study 5. ×265,000.

ferritin and other cell organelles such as the endoplasmic reticulum or the Golgi zone. Occasionally ferritin molecules were lying free in the cytoplasm (Fig. 7, opposite), but most were clearly membrane-bounded in cytosomes. Even in Study 2, where the haemoglobin concentration in the test medium was so low, ferritin could be easily seen in the cells.

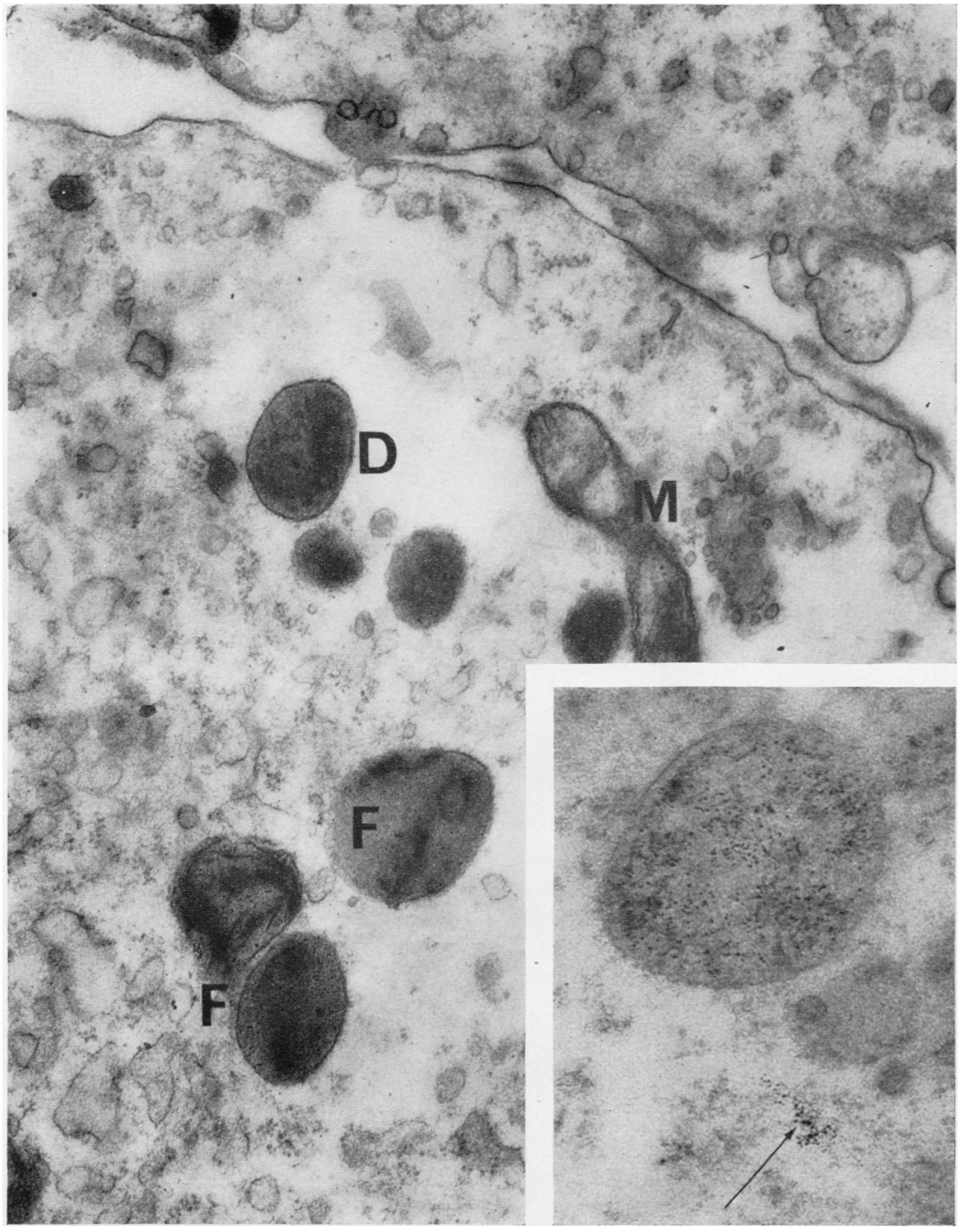
Discussion

These experiments have demonstrated that synovial cells in culture have the capacity to form ferritin in the presence of haemoglobin. Ferritin is very rare in erythrocytes (Bessis and Breton-Gorius, 1962), and in these experiments we did not find it in the haemoglobin residues outside the cell membrane. It is therefore quite unlikely that enough molecular ferritin was present with the haemoglobin added to the culture media to account for the amount seen in the cells. It is equally unlikely that iron in the synthetic culture media was a major factor in ferritin formation. Ferritin has not been found in control cultures in this and many other studies of cultured synovial cells grown in Medium 199. Iron in this medium is in the ferric form and it is believed that free molecular iron must be in the ferrous state for transport across cell membranes (Bothwell and Finch, 1962).

On the positive side we have found that ferritin appears around the haemoglobin particle within a vacuole or cytosome. The picture here is very

Fig. 6 —Membrane-bounded dense bodies are prominent within this cell. The cytosome (D) is free of granules but those indicated at F contain ferritin granules. Linear densities in one cytosome indicate a possible origin of this structure from a mitochondrion A mitochondrion is also indicated (M). Study 1. ×45,000.

Fig. 7 (inset).—Most of the ferritin in these cells is contained within cytosomes or vacuoles. Occasionally ferritin granules are seen lying free in the cytoplasmic matrix (arrow). Study 2. ×90,000.


D
M
F
F

similar to that seen in bone marrow during the phagocytosis and digestion of erythrocytes by reticular cells (Bessis and Breton-Gorius, 1962), where ferritin appears around fragments of red cells as they lighten in colour (compare Fig. 4).

The process of phagocytosis, intracellular transport, and digestion of haemoglobin have been well studied in the proximal tubule of the kidney (Miller, 1960; Ericsson, 1964, 1965). In these cells haemoglobin particles are taken up from the tubular lumen by phagocytosis. The vesicles and vacuoles containing haemoglobin move towards cytoplasmic areas containing the Golgi apparatus, mitochondria, and cytosomes. As in our study, there was evidence of fusion of cytosomes with the absorption droplets containing haemoglobin. Acid phosphatase was demonstrated within the cytosomes and it was assumed that enzymes capable of degrading haemoglobin were also present (Ericsson, 1965). During the next stage in both kidney and synovial cells the density of the haemoglobin particle decreases as ferritin granules appear. Miller (1960) concluded that the decrease in density of droplets indicated breakdown and removal of protein.

The appearance of ferritin implies that the cell has synthesized apoferritin, presumably under the stimulus of the iron released from the breakdown of haemoglobin (Richter, 1959). Richter has suggested that apoferritin might be synthesized in the vicinity of the endoplasmic reticulum. This is also present close to the ferritin deposits in the cells of the renal tubule. Ericsson (1964), in reviewing ferritin formation in renal tubules, considered that the synthetic processes also occurred within the cytosomes. We have noted some ferritin molecules free in the cytoplasm of synovial cells but a much larger proportion was membrane-bounded. We therefore consider it likely that the synthesis of ferritin and breakdown of haemoglobin take place within the same cytosome. Other tissues may show a different mechanism. Muir and Golberg (1961), in a study of subcutaneous macrophages after the local injection of iron dextran, considered that apoferritin was formed in relation to ribosomes and that iron diffused from the ingestion vacuole into the cytoplasm where ferritin was formed. They assumed that the ferritin then diffused back into the vacuoles. Richter, however, described findings similar to our own in experiments where ferrous sulphate was added to HeLa cell cultures. He noted that the ferritin first appeared in discrete membrane-bounded cytoplasmic bodies and that initially ferritin molecules were not dispersed through the cytoplasmic matrix (Richter, 1963). The concentration of iron used by Richter to produce this result was 5 μg/ml. medium, which is equivalent to the concentrations of haemoglobin iron used in this study.

It is interesting that such close similarities exist in the behaviour of cells so diverse as our connective tissue-derived cells and the HeLa and KB cells studied by Richter, which are permanently established lines of cells derived from human carcinomata. However, our chief interest in the present findings is that a simple explanation now exists for the presence of iron deposits in rheumatoid synovium. Although it is still possible that the iron comes from other sources, the rapid synthesis of ferritin from very low concentrations of haemoglobin suggests that the lysis of red cells is likely to be important.

Summary

The structure and behaviour of human synovial cells grown *in vitro* has been studied with the aid of the electron microscope. These cells in culture have the capacity to ingest haemoglobin prepared from haemolysed red cells. Ferritin appears concurrently in the cytoplasm.

The sequence and localization of these changes are described and compared with those observed in other tissues, and it is concluded that synovial cells are able to liberate iron from haemoglobin and to synthesize apoferritin.

The significance of these observations is discussed in relation to the accumulation of iron in rheumatoid synovial tissue.

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La formation de la ferritine par les cellules synoviales exposées à l'hémoglobine *in vitro*

RÉSUMÉ

On étudia la structure et le comportement des cellules synoviales humaines *in vitro* à l'aide du microscope électronique. Ces cellules en culture ont le pouvoir d'ingérer l'hémoglobine préparée à partir de globules rouges hémodialysés. La ferritine apparaît concurremment dans le cytoplasme.

On décrit l'ordre et le lieu de ces altérations et on les compare avec celles observées dans d'autres tissus. On conclut que les cellules synoviales sont capables de libérer le fer de l'hémoglobine et de synthétiser l'apoferritine.

On considère la portée de ces observations à l'égard de l'accumulation du fer dans le tissu synovial rhumatoïde.

La formación de la ferritina por células sinoviales expuestas a la hemoglobina *in vitro*

SUMARIO

Con la ayuda del microscopio electrónico se estudiaron la estructura y el comportamiento de las células sinoviales humanas *in vitro*. Estas células en cultivo tienen el poder de ingerir la hemoglobina preparada de eritrocitos hemodializados. La ferritina aparece concurrentemente en el citoplasma.

Se describen el orden y el lugar de estas alteraciones que se comparan con las observadas en otros tejidos. Se concluye que las células sinoviales tienen la capacidad de liberar el hierro de la hemoglobina y de sintetizar la apoferritina.

Se discute la importancia de estas observaciones en relación con la acumulación de hierro en el tejido sinovial reumatoide.