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IRON OVERLOAD IN CHANG CELL CULTURES: BIOCHEMICAL AND MORPHOLOGICAL STUDIES

A. JACOBS, T. HOY, J. HUMPHRYS AND P. PERERA

From the Department of Haematology, Welsh National School of Medicine, Cardiff

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Summary.—Cultures of Chang cells have been studied during growth in media supplemented with ferric nitriloacetate. Iron loading of the cells occurs rapidly and is related to the iron concentration in the medium. A 50-fold increase in cellular iron content was obtained in some cultures. Most of the intracellular iron is membrane-bound and is seen on electron microscopy to be concentrated in discrete bodies. There is a rapid rise in cellular ferritin content after exposure to iron. Most of this is found in the cytosol.

Iron taken into the cells is found equally in the cytosol and associated with membranes for the first 4 days of culture. After this time there is a rapid rise of membrane-bound iron associated with the formation of siderosomes which contain iron-rich ferritin cores. These siderosomes later evolve to contain irregular, electron-dense accumulations of iron.

Initial exposure of cells to high iron concentrations causes rapid death but similar exposure after ferritin synthesis and siderosome formation has been stimulated by low iron concentrations is well tolerated. Cultures have been maintained for up to 26 weeks with no morphological signs of toxicity, though there is some impairment of proliferation at high iron concentrations. It is suggested that siderosome formation is part of the mechanism that protects the cell against iron toxicity.

THE BODY lacks a specific physiological mechanism for the excretion of excess iron, normal iron balance being maintained through the regulation of absorption. Most of the iron in the body is present as circulating haemoglobin and much of the remainder is in storage form, either as the soluble protein ferritin or insoluble haemosiderin. An excessive iron load resulting either from abnormally increased absorption or through parenteral administration results in widespread tissue deposition with subsequent parenchymal damage and fibrosis. The pathology of iron overload has been described in detail (Sheldon, 1935; Cappell, Hutchison and Jowett, 1957; Oliver, 1959) and more recently there have been studies of the ultrastructural changes (Trump *et al.*, 1973; Iancu, Neustein and Landing, 1977).

Investigation of the iron overload state has been stimulated by its increasing occurrence following repeated blood transfusions in patients with chronic refractory anaemia or homozygous β-thalassaemia. In such cases the relief of anaemia is complicated by the inevitable complications of iron toxicity which eventually result in death of the patients, usually from cardiac or hepatic failure (Modell, 1976). Attempts to reduce the iron load in such patients by continuous chelation therapy have resulted in marginal biochemical improvement but little effect either on mortality or morbidity (Barry *et al.*, 1974). Despite much clinical interest in iron overload and its potentially fatal consequences, remarkably little is known about the intracellular process involved or the mechanism of iron toxicity (Jacobs, 1977*a*). Cellular iron uptake is known to stimulate ferritin synthesis (Harrison *et al.*, 1974) and this is thought to be a protective mechanism whereby iron is incorporated in

the ferritin shell in a non-toxic form. The nature of haemosiderin and its formation from ferritin is poorly understood (Jacobs, 1977*a*) and the role of lysosomes both in intracellular iron metabolism and in iron toxicity remains to be clarified.

Studies of cellular iron metabolism in cultured Chang cells have already been described (White, Bailey-Wood and Jacobs, 1976) and the present investigation is concerned with the possibility that these cells may be artificially iron-loaded and thus provide information on the resulting biochemical, structural and toxic changes occurring in this state.

MATERIALS AND METHODS

Chang liver cell (Flow Laboratories Ltd) were cultured in minimum essential medium (MEM) containing 10% (v/v) foetal bovine serum. The iron concentration of the basal medium was 6 μmol/l. Six sub-cultures were established in the same medium with additional iron in the form of ferric nitrilo-triacetate (Fe^{3+}NTA) at concentrations of 82, 161, 312, 454, 588, 714 μmol/l respectively. Ferric NTA was prepared by slowly dissolving ferric ammonium citrate (0·28 g) in 80 ml of water containing 0·382 g NTA and 0·9 g NaCl. The pH was adjusted to 7·0 with sodium bicarbonate, the solution made up to 100 ml and sterilized by Millipore (0·22 μm) filtration. Cells were subcultured every 7 days and some were harvested at this time. 15 ml of cell suspension containing about $15{\cdot}0 \times 10^6$ cells was centrifuged at 200 *g* for 5 min. The medium was discarded and the cells washed 3 times in 15 ml 0·9% NaCl. The cells were resuspended in 7·5 ml 0·9% NaCl and a sample (2·5 ml) of the suspension was removed. Cell numbers were determined using a Coulter Counter Model ZF and their viability estimated by Trypan Blue exclusion. The remaining 5 ml was sonicated for 30 s to disrupt the cells and 2·5 ml of the sonicate removed. The remaining sonicate (2·5 ml) was centrifuged at 20,000 *g* for 30 min to form a pellet and the supernatant was removed. The pellet was resuspended by sonication in 2·5 ml 0·9% NaCl. All fractions were stored at −20° while awaiting chemical analysis. After 6 weeks 2 subcultures of all cell lines were made into media containing 588 and 714 μmol/l iron respectively and then maintained in the same way as the other cultures.

Cells were fixed for electron microscopy with 3% glutaraldehyde in 0·1M sodium cacodylate–HCl buffer, pH 7·4, containing 1% sucrose. They were washed twice in the same buffer containing 7% sucrose and post-fixed in 2% osmium tetroxide. They were then dehydrated in graded ethanol solutions, embedded in Epon Resin and polymerized at 60°. 50–60 nm sections were cut on a Cambridge Huxley Ultramicrotome Mark II, mounted on copper grids and examined with a Phillips 300 electron microscope. When necessary, sections were double-stained with Reynold's lead citrate and uranyl acetate.

Iron was estimated with a flameless atomic absorption spectrometer (Varian Techtron Model 1100 with Model 63 carbon rod attachment). The samples were first sonicated (MSE 150 watt Sonicator, 40 s at an amplitude of 18 μm using a titanium pole). Both reference standards (0–10 μmol/l and samples were loaded in a volume of 2 μl, dry-ashed and atomized under the same conditions. Ferritin estimation was carried out by the method of Jones and Worwood (1975) using anti-human spleen ferritin antibody. All estimations were carried out either in duplicate or quadruplicate.

RESULTS

The cell cultures grown in media supplemented with iron at 82, 161, 312, 454 μmol/l grew in a normal manner and the cells harvested each week had a constant viability of about 70%. The cells initially cultured with iron added at a concentration of 588 and 714 μmol/l failed to grow. After the experiment had proceeded for 6 weeks, cells from each line were subcultured into concentrations of 588 and 714 μmol/l iron and from this time onwards they all continued to proliferate normally in these media after an initial growth retardation in the first week.

The mean total cell iron content before iron supplementation was 51 ± 29 fg per cell. 37 ± 26 fg per cell was membrane-bound and 17 ± 10 fg per cell was in the membrane-free supernatant. There was a sharp rise in cellular iron content following incubation in the iron-enriched media and this reached a plateau level after 1–4 weeks (Fig. 1). The mean iron concentration in the cells after the initial period was related to the level of iron supplementation (Fig. 2). Most of the intracellular iron was found associated with the membranous structures in the

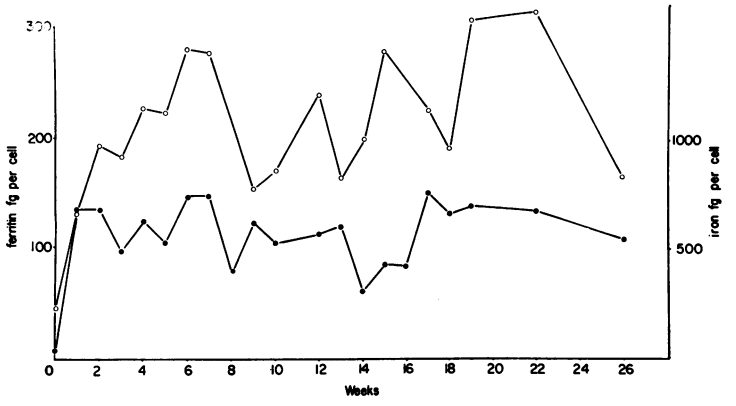

FIG. 1.—Total cell iron and ferritin content in cells incubated with 82 μmol/l iron from 0 to 26 weeks ●——● iron, ○——○ ferritin.

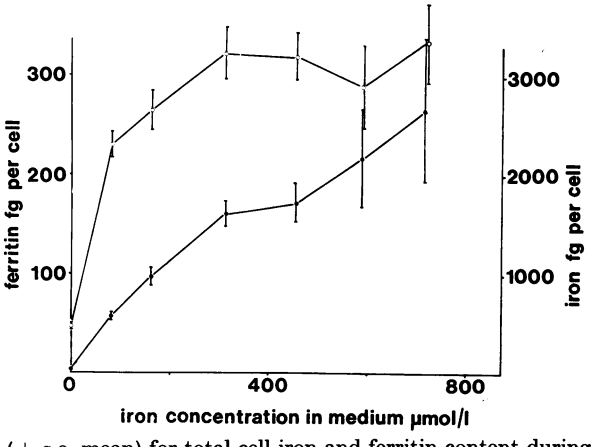

FIG. 2.—Mean values (± s.e. mean) for total cell iron and ferritin content during the period of 4–26 weeks' incubation with iron at concentrations of 5–714 μmol/l. Symbols as in Fig. 1.

pellet formed by 20,000 *g* centrifugation of the cell homogenate. This was always the case though, in those cells cultured in media containing 312 μmol/l iron or more, very high concentrations were found in the early part of the experiment but these tended to fall, the soluble iron in the cytosol remaining constant throughout (Fig. 3). When cells are first exposed to iron there is an increase in both cytosol and membrane-bound iron during the first 4 days but after this there is a rapid rise in membrane-bound iron (Fig. 4).

All cultures showed a marked increase in ferritin concentration after exposure to iron from a mean cell content of 46 ± 13 fg per cell under basal conditions to between 150 and 400 fg per cell after 1 week's incubation with iron. As in the case of iron content, the cellular ferritin content reached a maximum 1–4 weeks from the start of the experiment and thereafter remained high (Fig. 1). The maximum concentration of ferritin found in the cells is related to iron concentration in the incubation medium (Fig. 2), though at iron concentrations above 300 μmol/l there is no further increase in intracellular

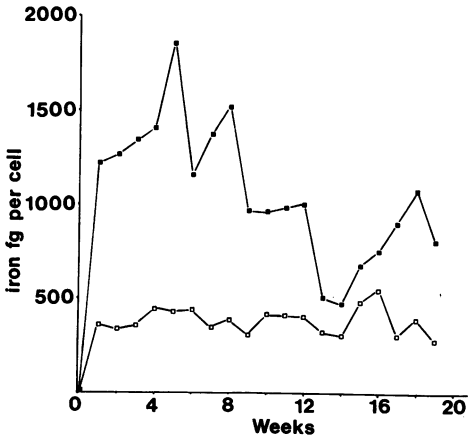

FIG. 3.—Iron concentration in membranes and supernatant of cells incubated with 312 μmol/l iron for 0–19 weeks. ■——■ membranes □——□ supernatant.

ferritin. The greatest rise in ferritin concentration occurs within 2 days of exposure to an abnormal iron concentration (Fig. 4).

Most of the intracellular ferritin measured by assay is found in the supernatant cytosol and very little is membrane-associated (Fig. 5). When cells which had grown in 454 μmol/l iron for 17 weeks were transferred to the basal medium containing no iron supplement, the initial ferritin content of 286 fg/cell had fallen to 56 fg/cell in 4 days and remained at that level. The total cellular iron content fell under these circumstances from 835 to 95 fg/cell in 1 week.

Electron microscopy shows the presence of dense iron cores of ferritin in the cytoplasm during the first 2 weeks after incubation with iron though even at this stage most of the electron-dense cores are associated with membranes (Fig. 6). Isolated iron-rich ferritin molecules are never visualized free in the cytoplasm after 3 or 4 weeks. Membrane-bound accumulations of dense iron cores are seen in increasing numbers after the third week (Fig. 7) and by the ninth and tenth weeks most cells have a large number of such bodies (Fig. 8). During the 4–8-week period a few small membrane-bound bodies of grossly irregular structure appear (Fig. 9), parts of which are electron-dense and parts lucent and apparently degenerate. After longer periods of culture these irregular amorphous deposits may increase in size and number. Electron probe analysis carried out by Mr J. Morgan, University College, Cardiff, using a Phillips 300 electron microscope with an EDAX (Energy Dispersive Analysis of X rays) attachment, shows both the more homogeneous deposits and the irregular deposits to have a high iron concentration.

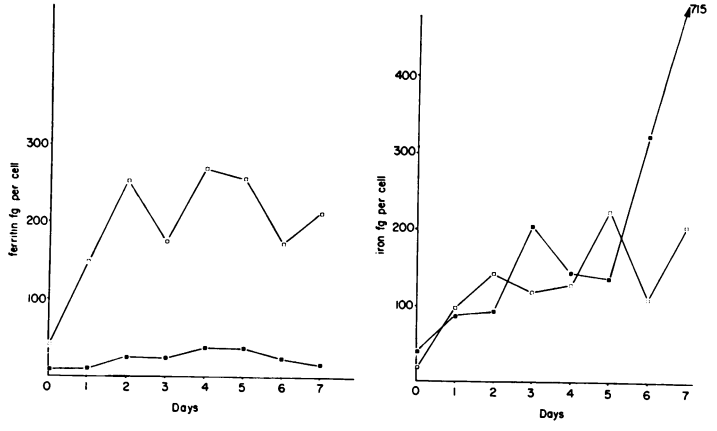

FIG. 4.—Ferritin and iron concentrations in membranes and supernatant of cells incubated with 161 μmol/l iron from 0 to 7 days. Symbols as in Fig. 3.

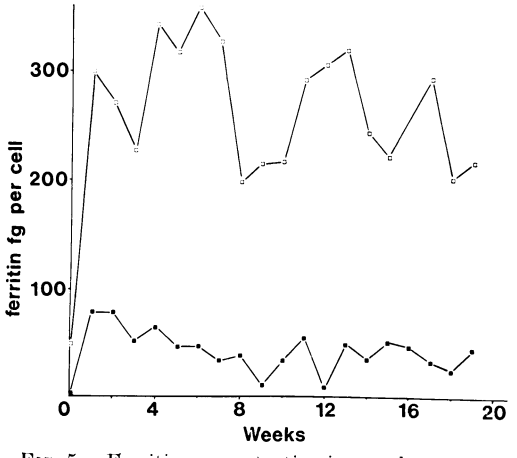

FIG. 5.—Ferritin concentration in membranes and supernatant of cells incubated with 312 μmol/l iron for 0-19 weeks. Symbols as in Fig. 3.

Although a few dying cells are seen in most cultures, most appear remarkably healthy despite containing large numbers of dense, iron-containing bodies. There was some reduction of cell numbers on subculture with increasing concentrations (Fig. 10), but no damage to organelles was seen and viability was apparently unimpaired. Cultures have been maintained in a satisfactory condition for up to 26 weeks.

DISCUSSION

Cultures of Chang liver cells provide an ideal model for the study of iron metabolism in non-erythroid tissue. Iron uptake in short-term cultures is followed by ferritin synthesis and the incorporation of iron into the ferritin protein (Bailey-Wood, White and Jacobs, 1975; White, Bailey-Wood and Jacobs, 1976). There is relatively little haem synthesis but an appreciable amount of the ^{59}Fe-labelled iron entering the cell can be found in a labile intermediate pool in the cytosol (White *et al.*, 1976; Jacobs, 1977*b*). The pool is available for uptake by ferritin and it can leave the cell either by binding to transferrin or an exogenous chelating agent. Ultrastructural studies of the liver in artificially iron-loaded animals (Trump *et al.*, 1973; Arborgh, Glaumann and Ericsson, 1974) or in human liver biopsy material (Iancu *et al.*, 1977) suggest that iron loading results in the accumulation of ferritin in lysosomes, at which site it is converted to haemosiderin. Unfortunately there is no clear definition of haemosiderin and the nature of this process is not clearly understood (Jacobs, 1977*a*). In the present study it has proved possible to examine some of the biochemical and cytological changes that occur when cultured cells become iron-loaded through constant exposure to an iron-rich medium.

The concentrations of iron used in the experiments varied from a level comparable to that found *in vivo* in the plasma of patients with gross iron overload to the maximum tolerated by the cultures without serious impairment of their growth rate. It was striking that all cultures appeared to be healthy throughout the experiments. Only occasional degenerate cells were seen and viability remained always at a constant level of over 70% before each subculture. Severe iron loading was consistent with a normal appearance of all organelles except for the accumulation of clearly defined siderosomes. The reduction in cell numbers with increasing iron concentration does not appear to be due to cell death but may be related to an impairment of proliferative capacity. The lowest iron concentration of 82 μmol/l used here can be obtained physiologically but all the others are higher than is found circulating in either clinical or experimental iron overload states. Iron uptake by Chang cells from transferrin is related to the degree of saturation of the protein with iron (Bailey-Wood *et al.*, 1975). In addition non-transferrin complexes in solution may also enter the cells (White and Jacobs, 1978). All cultures contained transferrin at 100% saturation and the additional Fe^{3+}NTA complex may be considered analogous to the non-transferrin iron said to be found in the serum of severely iron-loaded patients (Hershko and Rachmilewitz, 1976).

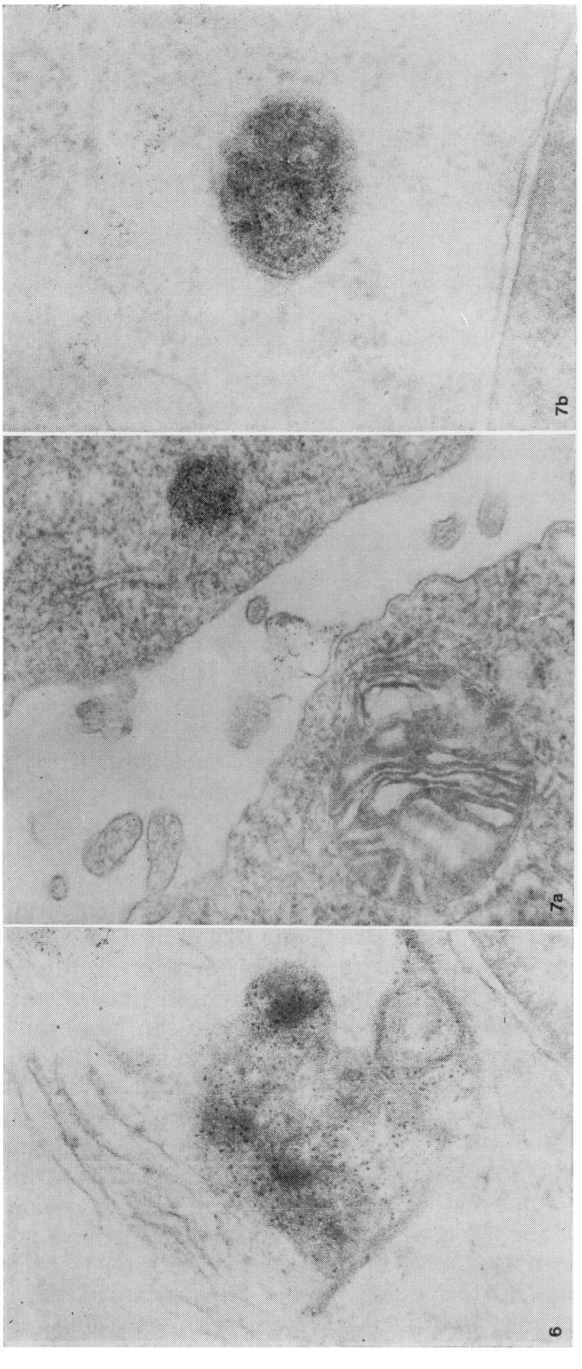

FIG. 6.—Discrete electron-dense cores associated with membranes after 1 week's incubation with 161 μmol/l iron. Unstained preparation (× 34,300).

FIG. 7.—Membrane-bound accumulations of dense iron cores after 2–4 weeks' incubation with 161 μmol/l iron. Unstained preparations (a × 34,300. b × 46,350).

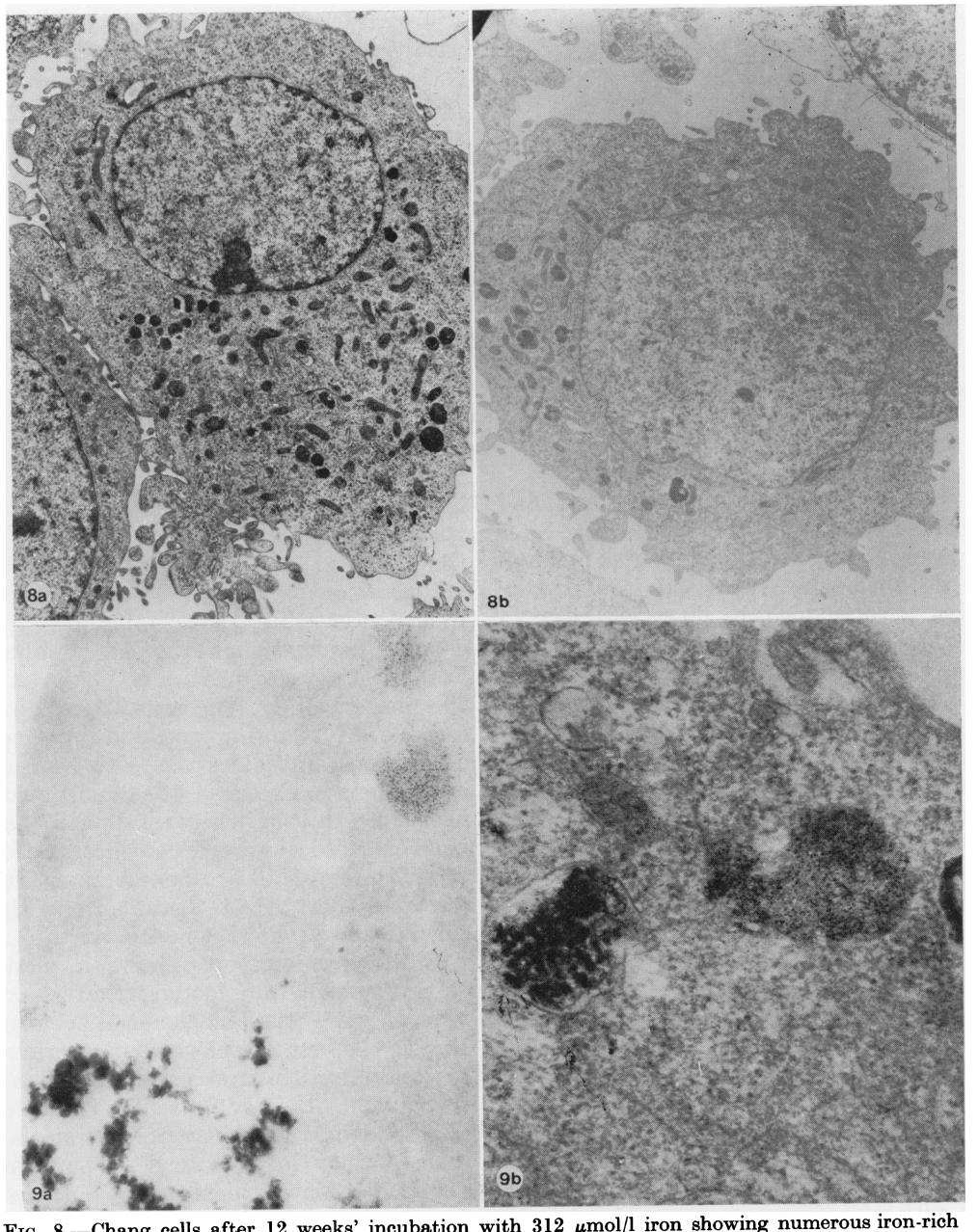

FIG. 8.—Chang cells after 12 weeks' incubation with 312 μmol/l iron showing numerous iron-rich bodies (× 6,120). (a) Stained with lead citrate and uranyl acetate; (b) Unstained.

FIG. 9.—Membrane-bound iron accumulations after 12 weeks' incubation with 161 μmol/l iron, some containing discrete ferritin cores and some containing irregular dense masses. (a) Unstained preparation (× 21,275); (b) Stained with lead citrate and uranyl acetate.

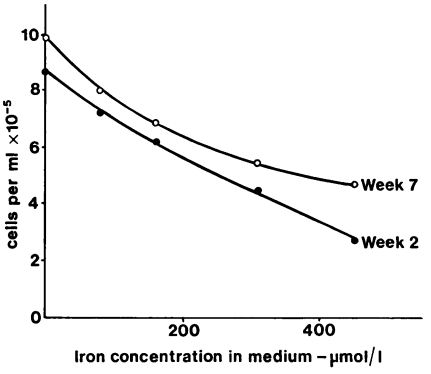

FIG. 10.—Number of cells in subculture after 10 days when grown at iron concentrations of 5–588 μmol/l.

The sharp rise in cellular iron and ferritin content in the first week after exposure to the iron-supplemented medium is not surprising, increased iron uptake being accompanied by increased ferritin synthesis. Earlier experiments (Bailey-Wood *et al.*, 1975) suggest that a maximum rate of ferritin synthesis is stimulated by an iron concentration of 50 μmol/l, slightly less than the lowest concentration used here. It might be expected that a constant rate of synthesis would either lead to a steadily rising ferritin content in the cell or that a balance between synthesis and degradation, or conversion to haemosiderin, would result in an equilibrium being attained. The ferritin content of the cells cultured in an iron-rich medium appears to reach a fairly stable plateau within a few days, most of the protein being found in the cytosol. Incubation in a medium containing more than 300 μmol/l does not lead to an increase in ferritin content, though iron content continues to rise (Fig. 2). The early response of the cell with regard to iron uptake appears to occur in 2 stages. During the first few days when acceleration of ferritin synthesis is at its maximum, iron is found equally in the cytosol and in membranous bodies, but by the end of the first week there has been an abrupt increase in membrane-associated iron, though only relatively little ferritin is found at this site. Although iron-rich ferritin may be more stable than apoferritin (Drysdale and Munro, 1966), it is also likely to enter lysosomes and be transformed to haemosiderin. In the initial process of degradation ferritin protein loses its immunoreactivity and can no longer be measured by the specific assay used here. Stimulation of lysosomal uptake by an increasing ferritin load might result in a relatively short life for an individual ferritin molecule and would account for relatively stable cytosol concentrations from the fourth week onwards and the rapidly increasing amount of iron in the cell pellet. The attainment of a relatively stable iron content after this initial period may be due partly to the halving of iron load with each cell division, which allows the burden to be shared by the daughter cells and possibly to cellular "defaecation" whereby the lysosomes eject their contents into the surrounding extracellular fluid, though the latter process has not been unequivocally observed in the present study. The cells appear to be capable of adapting to high iron concentrations. Initial exposure to iron concentrations in excess of 454 μmol/l results in cell death, but when cells which have been cultured at lower iron concentrations down to 82 μmol/l are later exposed to a concentration of 714 μmol/l iron, they survive and proliferate normally.

Electron microscopy shows a picture of progressive iron loading that is consistent with the biochemical changes. During the first 2 weeks of the experiment scattered ferritin iron cores can be seen throughout the cytoplasm. These are identified by their characteristic size and their density in unstained preparations. Single ferritin molecules are not easily found after this time, though from the first week onwards there are increasing numbers of siderosomes consisting of accumulations of iron cores. These are surrounded by a membrane, though this is not always obvious in unstained preparations. The siderosomes increase in number and undergo a gradual change in character with time. The earlier forms found at

about 2–3 weeks contain accumulations of ferritin iron cores (Fig. 7), but shortly after this the cores begin to present a more irregular appearance as degradation occurs (Fig. 9). Progressive changes within the siderosomes lead to the formation of larger electron-dense granules due to coalescence of the cores and as this process becomes advanced the iron deposits become less homogeneous, with dense iron masses scattered in an amorphous, somewhat translucent background. At 8–9 weeks all cells have large numbers of membrane-bound iron accumulations, some of which are still relatively homogeneous, but many of which form irregular deposits (Figs. 8 and 9). Despite the fact that these deposits have large lucent areas their overall iron content, in terms of their EDAX emission, is very high. While most cells showed marked siderotic changes at this stage there was little in their appearance to suggest damage to organelles.

The polymerization of ferritin molecules in solution has been recognized for some time (Williams and Harrison, 1968) and Niitsu and Listowsky (1973) have suggested that this process preferentially affects iron-rich molecules. With the incorporation of iron-rich ferritin into lysosomes, enzymatic degradation of the protein shell may be an important factor in the formation of haemosiderin, an insoluble substance with a high iron-to-protein ratio. The studies of Fischbach *et al.* (1971) suggest a similar atomic structure for the iron cores of ferritin and haemosiderin, which supports the view that one is derived from the other. Experimental iron overload in both rats and rabbits gives rise to lysosomal iron loading in the liver (Golberg, Smith and Martin, 1957; Trump *et al.*, 1973; Arborgh *et al.*, 1974) and similar changes are found in the pancreas (Pechet, 1969). In patients with transfusional iron overload, liver biopsy tissue shows iron accumulation confined to lysosomes (Iancu *et al.*, 1977) and in some cells amorphous iron deposits indistinguishable from those found in Chang cells can be seen (Iancu, personal communication). The mechanism whereby iron loading causes cell damage is still unknown. While increased lipid peroxidation with membrane damage has frequently been implicated (Lewis and Wills, 1962) with subsequent mitochondrial (McKnight, Hunter and Oehlert, 1965) and microsomal (Wills, 1969) damage, no evidence of these was found in the present study or in the other iron-loading animal studies mentioned here. In human biopsy samples from iron-loaded patients there is a significant increase in lysosomal enzyme activity and it has been suggested that these organelles are abnormally fragile (Peters and Seymour, 1976), though whether this might be a direct effect of chemically active iron on the lysosomal membrane or a mechanical effect of the massive deposits is not clear. We have no evidence regarding lysosomal enzyme activity in our cell cultures.

Iron loading in Chang cell cultures presents a cytological picture similar to that seen in both animal and human tissues. The absence of degenerative changes in cultured cells is, however, an important difference from the condition found in chronically iron-loaded liver. In culture the freely proliferating cells are able to reduce their iron content at each cell division and, in addition, there is an easy route for lysosomal excretion into the medium. This contrasts with the low rate of cell division by liver cells *in vivo* and the limited capacity of the organ to excrete iron. A more important difference between the cultured cells and iron-damaged organs in the body is the length of time during which the cells are exposed to high iron concentrations. The present study lasted for only 26 weeks and the cytological findings are comparable, though not identical, to those found by Iancu *et al.* (1977) in the liver parenchymal cells of young children, where during the first 2 years of life cellular overload was not accompanied by signs of cell death. While Peters and Seymour (1976) suggest that iron toxicity may be related to

lysosomal iron loading with subsequent lysosomal damage, two features of the present study suggest that visible iron deposits in the cell represent immobilized, metabolically inactive iron and that toxicity is a function of an intermediate labile pool (Jacobs, 1977*b*). Firstly, heavily loaded cells with dense iron-containing bodies show no signs of toxicity and have a normal viability. Secondly, cells which are normally killed by exposure to high iron concentrations are protected by initial stimulation of ferritin synthesis and haemosiderin formation following exposure to low iron concentrations. These appear to be protective activities and point to a labile iron intermediate being the direct cause of toxicity rather than lysosomal iron accumulation.

The present model appears useful as it lends itself to metabolic studies both of iron turnover and other biochemical activities. We have shown the pattern of iron and ferritin accumulation but further unanswered questions relating to intracellular pathways of iron metabolism, the mechanism of iron toxicity and the action of iron-chelating compounds on iron metabolism await study.

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