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Iron: Its Intracellular Localization and Possible Role in Cell Division*

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Abstract. When HeLa cells are fixed with ethanol, extracted with 0.1 N HCl, and incinerated at 540°C, all organic constituents and all ions studied are removed with the exception of iron. The gross outlines of cell structures are preserved and high concentrations of residual ash in interphase nucleoli and mitotic chromosomes suggest that there may be a shift in iron salts during the cell cycle. Experiments with cells isotopically labeled in proteins, nucleic acids, lipids, and polysaccharides indicate that the iron is bound to a polysaccharide. Addition of iron chelating agents to living cells causes a selective inhibition of DNA synthesis. These data suggest that iron may play a crucial role in the mitotic process.

Although inorganic elements are obviously essential in the cellular economy, their specific function in the mitotic process is unknown. Heilbrunn and others[1–4] have suggested that calcium may be linked to chromosome structure and condensation. We have recently shown[5] that hypertonic medium induces several mitotic-like phenomena in HeLa cells, including intranuclear condensation of chromosomes which at an ultrastructural level closely mimics that observed during prophase. The presence of Cu and Fe in chromosomes has been reported or suggested,[6–8] and chelating agents such as α,α-dipyridyl, as well as EDTA cause chromosome breaks;[9] however, the precise role of these elements in mitosis remains unexplored.

We have examined the intracellular distribution of iron throughout the cell. The interphase nucleolus and the metaphase chromosomes are particularly enriched depots, and iron may move from one site to the other at different phases of the cell cycle. Depriving HeLa cells of extracellular iron by adding an iron-chelating agent (Desferal, Ciba) rapidly inhibits DNA synthesis, and this in turn effectively prevents mitosis. In contrast, nondividing (contact-inhibited) diploid cells are unaffected by iron deprivation over a 4-day interval. It is of special interest that cellular iron binds preferentially to a material which can be labeled with isotopic glucose but not with amino acids, uridine, thymidine, or choline.

Materials and Methods. Cells: HeLa (S_3) cells were grown either as monolayers or suspension cultures in Eagle's medium supplemented with 7% fetal calf serum.[10] Synchronized cells in mitosis were obtained by selective detachment from monolayers as previously described.[11]

Microincineration: The basic method used is a modification of that described by Scott.[12] Cell monolayers were cultured to near confluence on 3 × 1 in. glass slides

kept at 100% humidity in an atmosphere of 5% CO_2, 95% air. The slides were washed in Ca^{2+}- and Mg^{2+}-free Earle's salt solution and fixed in 70% ethanol for 30 min, followed by 100% ethanol for 1 hr. They were then either dried directly or submerged in 0.1 N HCl for 30 min before rinsing and drying. The preparations were then placed in a Temco muffle furnace (Type 1500) at room temperature, gradually brought up to 540°C over a period of 6 hr, maintained at that temperature for at least 4 hr, and gradually cooled over a 4 to 6-hr period. The slides were immediately examined under phase contrast, in some cases after treatment with 0.1 N HCl as described under *Results*.

In order to monitor the completeness of incineration, monolayers on slides were labeled to equilibrium (72 hr) with one of the following: [^{14}C]leucine, [^{14}C]thymidine, [^{14}C]-uridine, [^{14}C]glucose, or [^{14}C]choline, each at a concentration of 0.5 μCi/ml. They were rinsed with Earle's salt solution, fixed with 5% trichloroacetic acid, washed with H_2O, and air dried. A $^3/_4$-in. square was cut from each of the labeled slides and the incorporated radioactivity measured in the Nuclear Chicago low background gas flow counter before and after incineration.

To determine the effects of the microincineration procedure on various ionic constituents of the cell, replicate suspension cultures containing 100 ml of cells at 2×10^5 cells/ml were labeled to equilibrium with either ^{45}Ca (10 μCi/ml), ^{59}Fe (5 μCi/ml), ^{65}Zn (10 μCi/ml), ^{63}Ni (1 μCi/ml), or ^{54}Mn (1 μCi/ml). Twenty-ml aliquots were centrifuged for the estimation of cell volume (or mass) (Bellco Glass, Vineland, N.J.). The supernatant fluid was saved for radioactive assay; after the wall of the tube above the pellet had been thoroughly rinsed, the cells were resuspended in 0.5 ml of H_2O, and mixed with 10 ml of Bray's solution for liquid scintillation counting. The total counts in the cell pellet were corrected for those present in the trapped extracellular fluid.[13] A second 20-ml aliquot of cell suspension was similarly measured after three washings in cold Earle's salt solution to assess readily exchangeable Fe, Ca, Zn, Ni, and Mn. A third aliquot was similarly prepared for scintillation counting after fixation in 70% ethanol (30 min) followed by 100% ethanol (1 hr); and a fourth aliquot was, in addition to fixation with ethanol, treated for 30 min with 0.1 N HCl. The effects of the ethanol treatment and of HCl extraction on Na, Mg, and K content were monitored on separate samples, using atomic absorption spectroscopy (Perkin-Elmer model 303).

Macromolecular locus of Fe binding in cell nuclei: To determine the macromolecular locus of iron binding in cell nuclei, 10^7 cells in 100 ml were labeled for 24 hr, with: (*A*) ^{59}Fe (25 μCi) and [^{3}H]leucine (100 μCi), (*B*) ^{59}Fe (25 μCi) and [^{3}H]thymidine (100 μCi), (*C*) ^{59}Fe (25 μCi) and [^{3}H]uridine (100 μCi), (*D*) ^{59}Fe (25 μCi) and [^{3}H]-glucose (100 μCi). Cell nuclei were prepared from all samples by Dounce homogenization in hypotonic buffer.[14] A small aliquot of each preparation was precipitated with trichloroacetic acid and assayed for radioactivity by double-channel liquid scintillation counting. A second aliquot of each was treated with a combination of DNase and RNase (100 μg/ml) in hypotonic buffer for 1 hour at 37°C, followed by Pronase (100 μg/ml) for another hour. Acetone or $CHCl_3$/methanol (2:1) was added to the residual pellet and the remaining pelletable material was washed with 10% trichloroacetic acid before addition of Bray's fluid for liquid scintillation counting.

Effects of iron depletion: Suspension cultures of synchronized cells in G_1, early S, late S, and G_2 phases of the cell cycle were treated with the iron chelating agent, Desferal mesylate (Ciba), at concentrations ranging from 0.05 mg/ml to 1.0 mg/ml, and the effects on initiation of DNA synthesis as well as on RNA and protein synthesis determined by pulse labeling 10^5 cells/ml for 15 min with 0.5 μCi/ml of [^{14}C]thymidine, [^{14}C]uridine, or [^{14}C]leucine as previously described.[11] Incorporation was stopped by adding cold Earle's salt solution (4°C), and the cells were pelleted, suspended in 5% trichloroacetic acid, and washed onto Millipore filters. The filters were dissolved in Bray's scintillation fluid and the incorporated isotope measured in a Beckman liquid scintillation counter. Entry of cells into mitosis was concomitantly followed in treated and untreated cultures by adding colchicine (0.1 μg/ml) and monitoring the accumulation of metaphase-arrested cells.

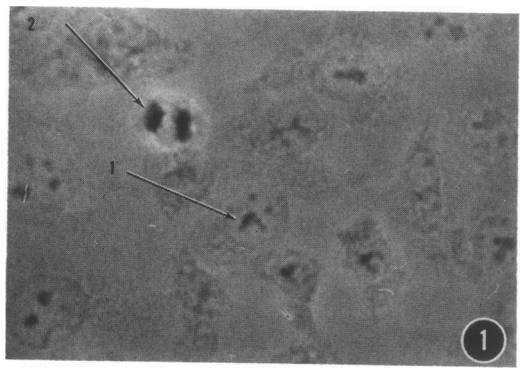

FIG. 1.—Phase-contrast micrograph of HeLa cell monolayer after incineration at 540°C for 4 hr. Monolayers were first washed in salt solution, fixed in 70% ethanol followed by 100% ethanol, and then incinerated. The outline of cell organelles remains visible with particularly dense concentrations of residue in interphase nucleoli and metaphase chromosomes. ×800.

Results. Localization of intracellular iron: The phase-contrast image in Figure 1 shows the residue of a HeLa cell monolayer after incineration at 540°C. The gross outlines of cell structures are remarkably well preserved, with the nucleoli in interphase (*arrow 1*) and the chromosomes in mitosis (*arrow 2*) particularly prominent. The interphase nucleoplasm is more diffuse, while the cytoplasmic residue is reticular. The residue is salt rather than uncombusted organic material (Table 1); thus when cell monolayers were labeled over several

TABLE 1. *Effects of incineration on incorporated macromolecular precursors.*

Macromolecular precursor	Trichloroacetic Acid-Precipitable Counts Before incineration	After incineration
[^{14}C]thymidine	12,500	4
[^{14}C]uridine	32,800	9
[^{14}C]amino acids	6,750	2
[^{14}C]choline	21,807	3
[^{14}C]glucose	65,200	6

Cell monolayers on 3 × 1 inch microscope slides were labeled to equilibrium with the indicated macromolecular precursors, washed in Earle's salt solution, fixed in ethanol, and counted; they were then incinerated at 540°C as described in the text and counted again.

generations with precursors of protein, DNA, RNA, phospholipid, or polysaccharide and then incinerated, there were no residual counts.

As shown in Figure 2, the ash dissolves in 0.1 N HCl. In contrast, when cells are fixed in ethanol and treated with 0.1 N HCl *before* incineration, some salts remain unextracted (Fig. 3). These are either partially or wholly iron deriva-

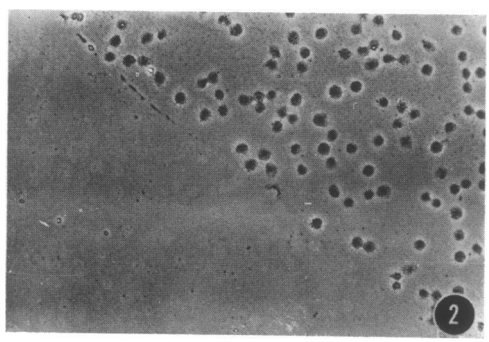

FIG. 2.—Phase-contrast picture of HeLa cell monolayer prepared as Fig. 1 except that after incineration the cells shown in the left half were treated with 0.1 N HCl. Note that all the residue has dissolved in the dilute acid. ×300.

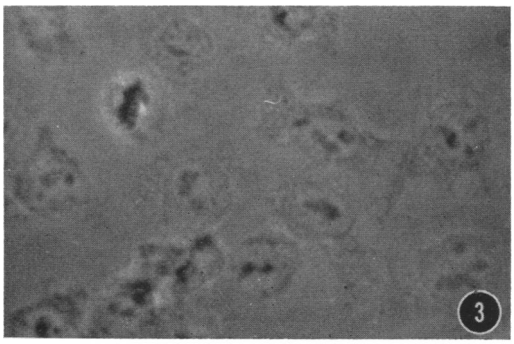

FIG. 3.—Phase-contrast photograph of HeLa cell monolayer prepared as in Fig. 1 but, in addition, treated with 0.1 N HCl *before* incineration. The residue in the nucleoli and the mitotic chromosomes remains prominent. ×800.

tives. Thus although cells labeled to equilibrium with ^{65}Zn, ^{45}Ca, ^{59}Fe, ^{63}Ni, or ^{54}Mn retain their radioactivity when fixed with ethanol, only ^{59}Fe counts survive extraction with 0.1 N HCl (Table 2). Mg, Na, and K also are removed by ethanol and HCl. Salts of other trace metals including Cu and Al remain to be investigated, but it is unlikely that they contribute significantly to the ash of Figure 3.

The ash of the interphase nucleoli and metaphase chromosomes in Figure 3 clearly contains significant amounts of inorganic iron salts. The data further suggest that during mitosis there may be a shift of iron, and perhaps other ions as well, from nucleolus to chromosomes. To rule out the possibility that the mitotic chromosomes only *seem* to have more iron salt residue simply because they are condensed, with more iron per unit volume, we have artificially induced chromosome condensation in interphase cells with hypertonic solutions as previously described.[5] The distribution of salt residue remains essentially the same as in untreated interphase cells (Figs. 4*a* and *b*).

The phase-contrast images of Figures 1–4 must, however, be interpreted with some caution. Perhaps 99% of nuclear constituents are eliminated by acid extraction followed by incineration (Tables 1 and 2); yet, under phase contrast the iron salts in nucleoli and chromosomes give these structures almost as much contrast as they show in living cells. This presumably reflects the relative phase effect of these structures with respect to their surroundings. There is little visible difference in the interphase nuclei of Figures 1 and 3, for example, even though most of the salts have been extracted from the nuclei of Figure 3. Conceivably, the proportion of salts extracted is the same in nucleoplasm as in nucleoli, so that the phase contrast remains the same.

TABLE 2. *Effect of preparation for incineration on intracellular cations.*

Treatment	Percentage of Maximum (Control)							
	Na	K	Mg	^{45}Ca	^{65}Zn	^{63}Ni	^{54}Mn	^{59}Fe
Control (unwashed cells)	100	100	100	100	100	100	100	100
Salt-washed cells	110	85	82	72	78	71	77	94
70% ethanol	6	7	78	70	73	67	69	91
0.1 N HCl	<0.1	<0.1	<1	<1	<1	<2	<1	77

Replicate suspension cultures were labeled to equilibrium with the indicated isotopes. They were then: (*A*) assayed directly for radioactivity, (*B*) washed with Earle's salt solution and assayed, (*C*) washed with Earle's salt solution, fixed with ethanol and assayed, (*D*) same as (*C*) but also treated with 0.1 N HCl. For details see text. In the case of Na, K and Mg data was obtained by atomic absorption spectroscopy.

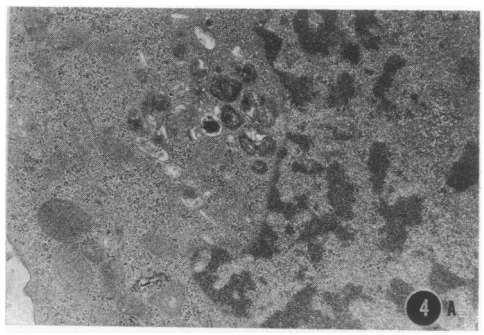

FIG. 4.—(*a*)Low power electron micrograph showing the "condensation" of interphase chromatin induced with a hypertonic solution.[5] HeLa cell monolayers were exposed to medium made 1.6× isotonic by the appropriate addition of crystalline NaCl. They were then fixed in 2.5% glutaraldehyde in the same hypertonic medium and processed for electron microscopy as previously described.[5] ×3000.

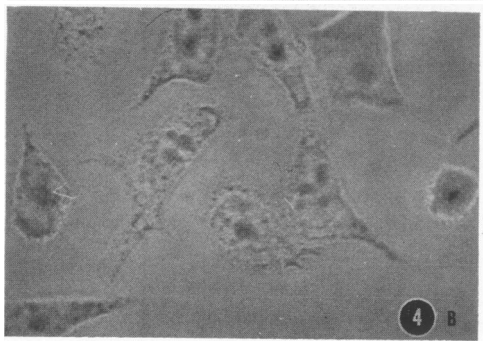

FIG. 4.—(*b*) Phase-contrast micrograph of HeLa cell monolayer prepared as in Fig. 1 except that the cells were exposed to 1.6× isotonic medium before fixation and incineration. Note that the artificial induction of chromatin "condensation" in interphase cells does not significantly change the distribution of nuclear ash. ×800.

Macromolecular binding of iron: By sequential enzymatic digestion of isolated nuclei we have determined that iron is bound preferentially to a macromolecule which incorporates glucose-^{3}H (or ^{14}C) but not thymidine, uridine, leucine, or choline. Table 3 shows that while DNase, RNase, and Pronase solubilized 90% or more of all trichloroacetic acid-precipitable counts in DNA, RNA, and protein, 84% of the ^{59}Fe counts remain acid precipitable. Acetone extraction, while removing 98% of the lipid, also has little effect on trichloroacetic acid-precipitable ^{59}Fe; in contrast, 60% of incorporated glucose-^{3}H remains precipitable along with the major fraction of ^{59}Fe. The implication is that the iron is associated with a polysaccharide. A crude pancreatic preparation (Viokase,

TABLE 3. *Effects of macromolecular degradation and extraction on nuclear trichloroacetic acid-precipitable radioactivity.*

	Percentage of Control					
Treatment	^{59}Fe	[^{3}H]-glucose	[^{3}H]-thymidine	[^{3}H]-uridine	[^{3}H]-leucine	[^{3}H]-choline
Control isolated nuclei	100	100	100	100	100	100
Nuclei after DNase, RNase, Pronase	84	60	7	8	5	95
Nuclei after enzymes and acetone	74	58	4	6	4	2

Five replicate cultures of HeLa cells each containing 4 × 10^{7} cells in 100 ml were labeled for 24 hr with 25 μCi ^{59}Fe plus *one* of the following: [^{3}H]glucose (100 μCi), [^{3}H]thymidine (100 μCi), [^{3}H]-uridine (100 μCi), [^{3}H]leucine (100 μCi), and [^{3}H]choline (100 μCi). The isolated nuclei from each nuclear sample were treated with: DNase (100 μg/ml) and RNase (100 μg/ml) for 1 hr, followed by Pronase (100 μg/ml) for 1 hr and acetone for 10 min. Each sample was then assayed for remaining trichloroacetic acid-precipitable radioactivity.

Nutritional Biochem.), which contains polysaccharidases, partially solubilizes the trichloroacetic acid-precipitable glucose-^{59}Fe counts, but 30% of the glucose counts still remain precipitable after 2 hr at 37°C.

In nuclear subfractions more than 50% of nuclear iron counts cosediment with the isolated nucleolar fraction. Although this is consistent with the distribution of salt residue noted in Figure 3, the possibility remains that it may reflect a tendency of the postulated polysaccharide to aggregate.

Effects of iron depletion on macromolecular synthesis and mitosis: The removal of iron from the medium by the addition of an iron-chelating agent, Desferal, inhibits DNA synthesis, regardless of the stage in the mitotic cycle at which the Desferal is added. Mitotic cells collected by selective detachment and immediately exposed to the drug never initiate the S phase, while cells treated at early or mid S show a rapid decline of [^{14}C]thymidine uptake over the following 2.5 hr (Fig. 5*a*); histone synthesis displays a parallel inhibition (not shown). Unlike those in S, cells treated in G_2 enter mitosis without lag. Thus when colchicine and Desferal are added simultaneously to randomly growing cultures, metaphase-arrested cells accumulate for the first 3 hr at precisely the same rate as in cultures treated with colchicine alone. This conforms to the experimental observation that, in contrast to DNA synthesis, RNA and protein synthesis continue at 70 and 85% of the control values, respectively, even 12 hr after Desferal addition (Fig. 5*b*).

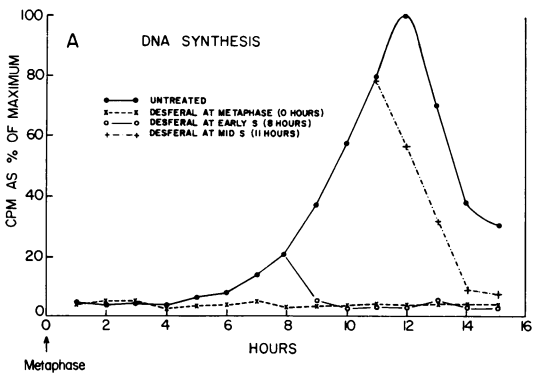

FIG. 5.—(*a*) Effects of Desferal on the synthesis of DNA in synchronized HeLa cells. Synchronized cells in mitosis were obtained by selective detachment, and Desferal (1 mg/ml) was added at various times thereafter as indicated. [^{14}C]-thymidine uptake into trichloroacetic acid-precipitable material was measured for one cell cycle.

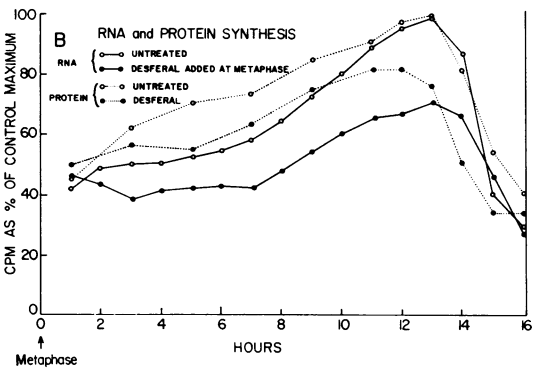

FIG. 5.—(*b*) Effects of Desferal on RNA and protein synthesis in synchronized HeLa cells. Synchronized populations were obtained and treated as in Fig. 5*a*. RNA and protein synthesis were measured by uptake of [^{14}C]-uridine and amino acids respectively into trichloroacetic acid-precipitable material.

In the light microscope treated HeLa cells remain indistinguishable from controls for at least 12 hr. By 24 hr there are signs of intracellular degeneration and considerable cell debris, with frequent intracytoplasmic accumulation of refractile granules, and by 48 hr the majority of cells have disintegrated. However, addition of 1 mg/ml Desferal to contact-inhibited diploid cells causes no obvious morphological changes over a 4-day period.

Table 4 illustrates the effects of Desferal on the uptake of ^{59}Fe, ^{45}Ca, ^{65}Zn, ^{22}Na, ^{63}Ni, and ^{54}Mn. ^{59}Fe is the only isotope affected, and here uptake is completely prevented, suggesting that the cellular response to Desferal is related to the drug's selective affinity for iron. However, the unlikely possibility cannot be excluded that Desferal *per se* has a unique toxic effect independent of its affinity for iron. It should be noted that since iron is not usually added to cell culture medium, the necessary amounts must be present as trace contaminants.

TABLE 4. *Effects of Desferal on cation uptake.*

Ion	Control (cpm)	Desferal (cpm)
^{22}Na	5,780	5,680
^{45}Ca	15,600	16,700
^{63}Ni	1,340	1,100
^{54}Mn	4,620	4,800
^{65}Zn	22,500	21,060
^{59}Fe	14,600	130

12 replicate cultures containing 75 mg of HeLa cells in 25 ml of spinner medium were prepared. Desferal (1 mg/ml) was added to six of these. 5 min later, each of the indicated isotopes was added to one treated and one untreated culture (^{22}Na, 2 μCi/ml; ^{45}Ca, 10 μCi/ml; ^{63}Ni, 1 μCi/ml; ^{54}Mn, 1 μCi/ml; ^{65}Zn, 2 μCi/ml; ^{59}Fe, 5 μCi/ml). After 2 hr the samples were spun, without washing, into centrifuge tubes specially designed for measuring cell volume, and the radioactivity in the cells versus the extracellular fluid was estimated as described elsewhere.[13]

Discussion. The present experiments imply a hitherto unsuspected role for iron in the cell economy. They indicate that the nucleolus is a repository of interphase nuclear iron, and that this may be transferred to the chromosomes during mitosis. Experiments involving enzymatic degradation and lipid extraction indicate that most of the nuclear iron is bound to a polysaccharide. The complexing of iron and polysaccharide in the HeLa cell is one example of a well-known biological phenomenon and "the early literature on colloids is filled with references to complexation of iron with gum arabic and other polysaccharides."[15] More recently Davis *et al.*[16] have isolated a glycoprotein from gastric juice which tenaciously binds iron making it unavailable for uptake. The absence of this compound from gastric juice results in uncontrolled absorption of dietary iron leading to hemochromatosis. Fletcher and London[17] have shown that an iron-dextran complex is clinically useful in the intravenous administration of the metal, again presumably allowing controlled utilization by the body. The precise role played by the iron-polysaccharide complex in HeLa is not clear. It may be that as postulated for gastroferrin, there is controlled release of iron from the complex which might in turn mediate chromosome condensation, but further study of this point is obviously necessary. Finally, with the necessary reservations indicated above as to the mode of action of Desferal, the data sug-

gest that iron plays a crucial role in the initiation and maintenance of DNA synthesis.

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