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Intracellular Iron-Binding Macromolecules in HeLa Cells

(chelation/polysaccharide/protein/DNA synthesis/desferrioxamine)

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ABSTRACT The concentration of the iron-chelating agent, desferrioxamine (Desferal), that just inhibits iron entry into HeLa cells is also the concentration that inhibits DNA synthesis. As a first step in clarification of the mechanism whereby iron may partake in DNA synthesis, we have partially characterized several of the intracellular iron-binding sites. Most cytoplasmic iron appears to be bound to a polysaccharide containing glucose that sediments at about 32 S. Nucleolar iron is bound to a single protein, the mobility of which is independent of the concentration of sodium dodecyl sulfate in an acrylamide gel. In contrast the pattern and mobility of nuclear iron, other than nucleolar, is heterogeneous and markedly affected by the concentration of sodium dodecyl sulfate. The evidence suggests that nuclear iron is bound to protein through one or more intermediate(s).

Addition of the iron-chelating agent, desferrioxamine (Desferal, Ciba), to HeLa cells selectively prevents entry of ^{55}Fe (as citrate) and reversibly inhibits DNA synthesis, with little effect on RNA or protein synthesis (1). In the present paper we note that the concentration of desferrioxamine that just inhibits entry of iron into cells is precisely that concentration that also causes cessation of DNA synthesis, implying that perpetual addition or replacement of iron is critical for cell division. Thus it is of interest to characterize the intracellular iron-binding sites in HeLa cells since one or more of them may function in the regulation of DNA synthesis. Granick (2) has suggested that ferritin is the principal iron-binding site in organs such as spleen and liver, while Wacker and Vallee (3) have given evidence indicating that most tissue iron is bound to RNA. Eichorn (4) showed that Fe^{++} stabilized hydrogen bonds in solutions of calf-thymus DNA and felt that the metal probably complexed with PO_4^{-3} groups. We here present data suggesting that, in contrast to the findings of Wacker and Vallee, most *cytoplasmic* iron is bound to polysaccharide(s); *nuclear* iron, on the other hand, appears to be bound to several proteins, possibly by way of an intermediate. Although nucleoplasmic iron has a heterogeneous distribution on acrylamide gels, nucleolar iron is bound to a single protein. Thus, while most body iron is undoubtedly bound to certain long-recognized repositories such as heme, transferrin, ferritin, and various enzymes, e.g., cytochrome oxidase (5), it now appears that a significant fraction has a previously unsuspected distribution and the macromolecules involved probably have functions yet to be elucidated.

MATERIALS AND METHODS

Cells. HeLa cells, subline S_3, maintained in Eagle's suspension culture medium (6) supplemented with 7% fetal-calf serum were used throughout.

Effects of Desferrioxamine Concentration on Macromolecular Synthesis. Aliquots containing 50 ml of logarithmically growing HeLa cells (2×10^5 cells per ml) in suspension culture were treated with desferrioxamine at concentrations ranging from 0–1 mg/ml. Uptake of [^{14}C]thymidine, [^{14}C]aminoacids, and [^{14}C]uridine into trichloroacetic acid-precipitable material was monitored hourly during a 10-hr interval by methods already described (7).

Effects of Desferrioxamine Concentration on Cellular Uptake of ^{55}Fe. A correlation between the effects of desferrioxamine concentration on macromolecular synthesis and on ^{55}Fe uptake was made. ^{55}Fe as ferrous citrate (New England Nuclear) was added to aliquots containing 50 ml of logarithmically growing HeLa cells (2×10^5/ml) in suspension culture, so that the final isotope concentration was 1 μCi/ml. 5 Hours later, desferrioxamine was added to final concentrations ranging from 0–1 mg/ml. Trichloroacetic acid-precipitable intracellular iron counts were determined at hourly intervals during a 4-hr period.

Distribution of Bound Iron in Subcellular Fractions. Individual experiments required different quantities of cells and different isotope concentrations, but in general about 10^8 cells in 500 ml of complete medium were labeled for 3 hr with 1 mCi of ^{55}Fe citrate. The pH of the suspension was monitored continuously and maintained at 7.0. The cells were collected by centrifugation and washed four times in Earle's spinner salts to insure removal of all exogenous iron label. The final pellet was resuspended in 30 ml of hypotonic Tris buffer (7), swollen for 10 min, and lysed with 20 strokes of a tight-fitting Dounce-type homogenizer. Nuclei were pelleted at 2500 rpm for 1 min in the PR 2 International centrifuge (253 rotor) Cytoplasm was removed and saved. The nuclear pellet was washed three times in hypotonic buffer (2500 rpm for 30 sec). In some experiments the nuclei were further purified by centrifugation through 2 M sucrose, but this did not significantly improve the final preparation. Nucleoli were prepared by a modification of Busch's procedure (8, 9): A fraction of the washed nuclear sample was resuspended in hypotonic buffer containing 1 mM $CaCl_2$ and sonicated (Branson sonifier, setting 6 for 2 min with the microtip probe). This schedule disrupted about 98% of nuclei and had little observable effect on nucleoli, which maintained their morphology as irregular, refractile bodies. The sonicate was diluted 1:2 with Ca^{++}-free hypotonic buffer, giving a final Ca^{++} concentration of 0.33 mM. The nucleoli were easily separable from the remaining

Abbreviation; SDS, sodium dodecyl sulfate.

TABLE 1. *Uptake of ^{55}Fe (cpm) as a function of concentration of desferrioxamine*

	Desferrioxamine concentration (mg/ml)				
Hr	0	0.05	0.2	0.5	1.0
0	1110	1100	1170	1090	1180
1	2010	1210	1300	1170	1200
3	4200	1400	1310	1080	1100
5	6400	1700	1590	1100	1170
7	8100	1810	1600	1270	1090
9	10000	2200	1580	1100	1170

nuclear constituents by differential centrifugation (2500 rpm for 8 min in the PR 2 International centrifuge, 253 rotor, followed by suspension and centrifugation, (three times, at 2000 rpm for 5 min). Supernatant from the first spin, containing nucleoplasm and fragmented nuclear membranes, was saved.

Representative aliquots of whole cell suspensions, cytoplasm, nuclei, and nucleoli were diluted with 5% Cl_3CCOOH filtered onto Millipore discs, and counted in Bray's solution in a Beckman liquid scintillation counter model 230.

Cytoplasm was further fractionated into a 100,000 × *g* supernatant and a boiled supernatant fraction. In addition, polyribosomes were assayed for bound iron by layering 1.5 ml of cytoplasm on 15–30% linear sucrose gradients and processing as described (10). Both 100,000 × *g* supernatant and boiled supernatant were analyzed on sucrose and CsCl gradients. Sucrose gradients of several different compositions were run for various periods of time, and acid-precipitable iron counts were assayed. We determined that a 7–20% linear gradient spun at 41,000 rpm for 200 min in an SW41 rotor delineated cytoplasmic iron counts effectively. This procedure was also used to prepare milligram quantities of iron-binding material: Cytoplasm from 50–100 g of ^{55}Fe-labeled HeLa cells was concentrated 10-fold before it was layered on 7–20% sucrose gradients, and the peak ^{55}Fe fractions from six gradients were pooled. These were dialyzed against distilled water (20 liters) for 12 hr, concentrated to 1 ml against Carbowax 6000 (Union Carbide), and spun again on sucrose gradients. After a second dialysis and concentration, the material was passed through Sephadex G100 with H_2O as the eluent. The void volume eluate was then lyophilized, and could be stored indefinitely. For further analysis, 1 mg was dissolved in 1 ml of 1 N HCl, and hydrolyzed at 100° for 12 hr. The hydrolysate was assayed for ninhydrin-positive material and reducing sugars by chromatography with appropriate standards. For the reducing sugars, the solvent and development system of Trevalyan (11) was used. In some cases the partially purified material from sucrose gradients was mixed with CsCl (1 g/ml of hypotonic buffer) and centrifuged for 48 hr at 55,000 rpm. Iron-binding material was pooled from peak gradient fractions, dialyzed against 20 liters of distilled water, and treated as above. In some experiments samples were treated with DNase, RNase, Pronase, and α- and β-amylase (1 hr each) before centrifugation.

Nuclear ^{55}Fe. Nuclei or isolated nucleoli, or both, in hypotonic buffer were treated with DNase and RNase for 60 min at 37° (50 μg/ml final concentration) and solubilized in 1% sodium dodecyl sulfate (SDS) (final concentration). Insoluble debris was removed by centrifugation, and the supernatant was electrophoresed on polyacrylamide gels. The Maizel neutral SDS–acrylamide gel buffer (Table 1 of ref. 12) was modified so that both sample buffer and gel buffer were 10 mM Tris·HCl, pH 7, thus eliminating the usual concentration step at the sample/gel interface. The reservoir buffer was 0.1 M Tris·HCl–0.1% SDS. In all other respects the system was as described by Maizel (12). Elimination of the concentration step at the gel/sample interface was crucial for these experiments. When the ratio of the concentrations of sample buffer to gel buffer was 1/10, as is usual, all bound ^{55}Fe was released during electrophoresis and was detected as a single sharp peak of Cl_3CCOOH-soluble material.

RESULTS AND DISCUSSION

Effects of desferrioxamine concentration on macromolecular synthesis and ^{55}Fe uptake

We have previously shown that at concentrations of 1 mg/ml desferrioxamine inhibits DNA synthesis, but not RNA or protein synthesis. The purpose of the present experiments was to show that the lowest concentration that just inhibits DNA synthesis is precisely that concentration that completely blocks the uptake of ^{55}Fe. Tables 1 and 2 illustrate that at all desferrioxamine concentrations above 0.05 mg/ml both DNA and iron uptake cease, while RNA and protein synthesis are only slightly affected. The precise correlation between the desferrioxamine concentration that inhibits iron uptake and that which inhibits DNA synthesis strongly suggests a causal relationship, especially since desferrioxamine does not affect transport of any other ion tested (1). However, while des-

TABLE 2. *Macromolecular synthesis (cpm) as a function of concentration of desferrioxamine (mg/ml)*

	1			0.5			0.2			0.05			0		
Hr	dT	U	RPH	dT	U	RPH	dT	U	RPH	dT	U	RPH	dT	U	RPH
1	2500	3600	1700	3700	3400	1590	4500	3600	1850	4800	3700	1650	4800	3300	1700
2	1500	3900	2010	2200	4000	1900	4600	4000	2200	5200	4400	2000	5100	4200	2100
3	340	4000	1800	706	4200	2100	3800	4300	2200	5500	4500	2100	5400	4400	2200
4	106	3000	1900	170	4200	2300	2500	4600	2400	3900	4500	2300	5800	4700	2400
5	94	4200	2000	120	4300	2300	2300	4900	2900	3200	4800	2400	6400	4900	2900
6	104	4100	2400	100	4400	2600	1900	4960	3100	3000	4900	2700	6700	5000	3000
7	109	4600	2600	106	4600	3000	1100	5000	3000	2800	5800	3100	7100	5200	3000
8	92	4700	2800	94	4900	3300	200	5300	3200	2200	5400	3400	7900	5700	3100

RPH, reconstituted protein hydrolysate.

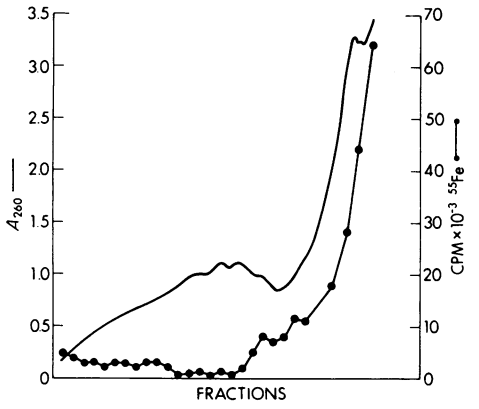

FIG. 1. Distribution of ^{55}Fe on cytoplasmic polysomes. Cells were labeled with ^{55}Fe citrate. Cytoplasm was prepared and layered on 15–30% sucrose gradients, spun at 24,000 rpm for 2 hr in an SW25 rotor, and assayed for A_{260} and trichloroacetic acid-precipitable counts.

ferrioxamine concentrations of 0.2 mg/ml and higher block ^{55}Fe uptake instantly, cessation of DNA synthesis requires various intervals, which are dependent on drug concentration. Thus, at 1 mg/ml desferrioxamine arrests thymidine incorporation within 3 hr, but at 0.2 mg/ml 8 hr is required. These data imply that, besides blocking iron uptake, desferrioxamine acts intracellularly as well, possibly disrupting the function of iron-containing macromolecules involved in DNA synthesis. It would be expected that the efficiency of this disruption would be dependent on intracellular drug concentration, which in turn is probably a function of extracellular concentration.

Fe distribution in subcellular fractions

Table 3 illustrates the subcellular distribution of isotopic iron after a 3-hr pulse. About 71% of the acid-precipitable counts are in the cytoplasm and 17% are in the nucleus; the remainder are lost during processing. About 65% of total cytoplasmic counts remain in the supernatant fraction after 20 min at 100° and, similarly, 65% of the counts are in the 100,000 × *g* supernatant. The nucleolus contains about 1–2% of the total incorporated counts. This is not artifactual, as shown below. When longer pulses were used or when cell concentration was varied, the distribution was somewhat different, but the pattern shown here is a reasonable generalization.

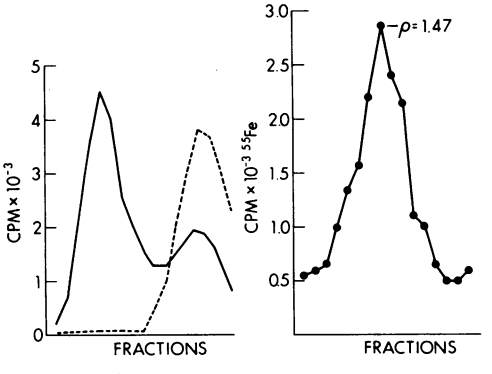

FIG. 2. (*Left*) ^{55}Fe distribution in cytoplasm. Cytoplasm labeled with [^{14}C]lysine and ^{55}Fe citrate was layered on 7–20% sucrose gradients and spun at 41,000 rpm in an SW41 rotor for 4 hr. ——, ^{55}Fe; – – – [^{14}C]lysine. Fractions were monitored for acid-precipitable counts.

FIG. 3. (*Right*) Distribution of cytoplasmic ^{55}Fe on CsCl gradients. Cytoplasm was treated with DNase, RNase, Pronase, and β-amylase, mixed with CsCl, and spun at 55,000 rpm in an SW65 rotor for 48 hr. Fractions were monitored for acid-precipitable activity.

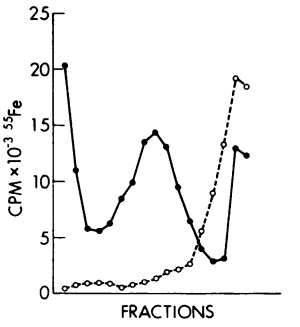

FIG. 4. Iron-binding material in the nucleus. ^{55}Fe-labeled nuclei were treated with DNase, RNase, and 1.0% SDS. The solubilized material was layered on 7–20% sucrose gradients containing 1.0% SDS and spun in a type 65 rotor at 60,000 rpm for 3 hr (O– – –O) and for 30 hr (●——●). Fractions were assayed for acid-precipitable radioactivity.

When cytoplasm was layered on 15–30% linear sucrose gradients and processed for bound iron on polyribosomes none was found attached to either polysomes or single ribosomes (Fig. 1); most of the acid-precipitable counts remained at the top of the gradient, eliminating ribosomal, messenger, and 5S RNA as iron-binding sites.

When boiled cytoplasm labeled with ^{55}Fe and [^{14}C]lysine was layered on 7–20% linear sucrose gradients and spun at 41,000 rpm for 200 min, a single sharp peak of iron-binding material sedimenting at about 32 S was detected (essentially all lysine counts and a small fraction of ^{55}Fe counts remained at the top of the gradient) (Fig. 2). Untreated cytoplasm

TABLE 3. *Intracellular distribution of ^{55}Fe*

Fraction	cpm
Whole cell	3,590,000
Cytoplasm	2,560,000
Boiled cytoplasm	1,620,000
100,000 × *g* supernatant	1,650,000
100,000 × *g* pellet	700,000
Nuclei	606,000
Nucleoli*	63,000

Cells were labeled and fractionated as described in the text. Aliquots from each fraction were precipitated with 5% Cl_3-CCOOH and filtered onto Millipore filters. These were treated with 1 ml NCS–H_2O 9:1 (Amersham). After 4 hr, 20 ml of Bray's fluid was added.

* The fraction of bound intracellular iron originally reported as "nucleolar" (1) was higher than that noted in the present paper. Since this earlier report, we have found that ^{55}Fe binds avidly to certain dense inorganic material in spinner cultures; this material was originally isolated with nucleoli. This problem has been eliminated by assaying only the upper half of the nucleolar pellet. The lower half contains a gray material that is easily recognized and avoided. Since this material is not soluble in SDS it does not run in SDS gels and, therefore, does not contribute to the results shown in Figs. 5–8.

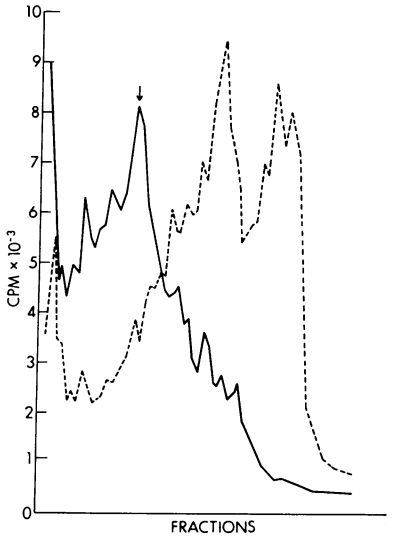

FIG. 5. Iron-binding material in the nucleus. Nuclei labeled with [^{14}C]lysine or ^{55}Fe were treated as in Fig. 4 and electrophoresed on 7.5% polyacrylamide gels containing 0.1% SDS for 4 hr at 10 mA/gel. ——, ^{55}Fe; – – –, [^{14}C]lysine.

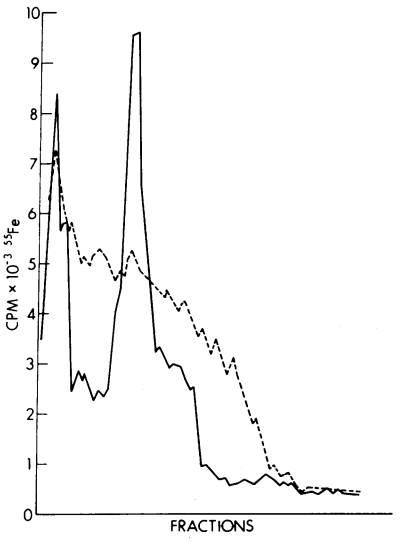

FIG. 6. ^{55}Fe in nucleoli and nucleoplasm. ^{55}Fe-labeled nucleoli (——) and supernatant from nucleolar isolate (– – –) were treated as in Fig. 4 and electrophoresed on 7.5% polyacrylamide gels as in Fig. 5.

gave the same shape curve except that significant counts were pelleted, as would be expected from Table 3. In this paper we will not be concerned with those iron counts in the 100,000 × *g* pellet. Neither the shape nor the height of the cytoplasmic iron peak was affected by RNase, DNase, Pronase, or α- or β-amylase.

Isolation and purification of the iron-binding material from sucrose and CsCl gradients yielded a slightly orange, powdery substance that was readily soluble in H_2O, but less so in 1 N HCl. The peak of iron-binding material recovered from CsCl gradients (Fig. 3) had a density of 1.47 g/ml. This established that it probably was not RNA, DNA, or protein, all of which have markedly different densities; on the other hand, some polysacchrides fall within this range. It, therefore, was not surprising to find that upon hydrolysis, chromatography, and silver staining, a single intense spot with the same R_f as glucose was found. Gas–liquid chromatography confirmed identification of the spot. We, therefore, suggest that cytoplasmic iron may be bound to a polysaccharide containing, at least, glucose. Although we have not ruled out the possibility that both the iron-binding material and polysaccharide are separate entities that have the same density, this does not seem likely. It is quite possible that the cytoplasmic iron-binding polysaccharide is glycogen in spite of its insensitivity to α- and β-amylase; this insensitivity may merely indicate that enzyme sites are blocked by the presence of iron or that it is a special form of glycogen.

Analysis of bound nuclear iron required dissolution of the nucleus and nucleolus. This was most effectively accomplished with 1.0% SDS after treatment with DNAse and RNase. When dissolved whole nuclear samples were layered on 7–20% sucrose gradients and spun for 200 min as described for cytoplasmic samples, all acid-precipitable material remained at the top of the gradient, in contrast to the pattern obtained with cytoplasm; however, a peak of iron-binding material did pass into the gradient when centrifugation time was increased to 30 hr (Fig. 4). In addition some nuclear counts were pelleted. Nuclear iron-binding material was characterized in more detail with polyacrylamide gel electrophoresis of nuclei labeled with iron and lysine. Unlike bound cytoplasmic iron, essentially all of which resisted migration into 7.5% gels, most nuclear iron was heterogeneously dispersed through the beginning half of the gel (Fig. 5). The most rapidly migrating, lysine-rich peak at the end of the gel that contains arginine-rich histones (10) was singularly free of bound iron. In fact, subsequent to the most conspicuous Fe^{++} peak (*arrow*), there was a general decrease in the bound iron/lysine ratio all along the remaining length of the gel.

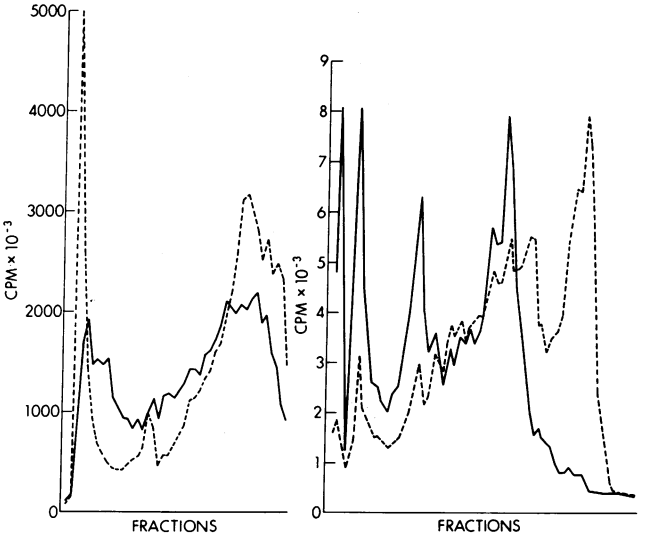

FIG. 7. (*Left*) Effects of trypsin on bound ^{55}Fe in nuclei. Nuclei labeled with [^{14}C]lysine or ^{55}Fe were treated with DNase, RNase, and then trypsin (0.1%) for 1 hr. Nuclei were solubilized in 1.0% SDS and electrophoresed as in Fig. 5. ——, ^{55}Fe; – – –, [^{14}C]lysine.

FIG. 8. (*Right*) Effects of increased gel SDS concentration on the migratory patterns of nuclear iron-binding material. Nuclei labeled with ^{55}Fe or ^{14}C lysine were prepared and electrophoresed as above, except that the gel and the circulating buffer concentration of SDS was increased from 0.1 to 1.0%.

The indicated peak in the whole nuclear sample (*arrow*, Fig. 5) is of nucleolar origin as shown by Fig. 6, which is an electropherogram of iron-labeled nucleoli and nucleolar supernate. The single iron-rich peak from nucleoli has the same mobility as the most prominent peak in the whole nuclear fraction; furthermore, when the nucleoplasm (supernatant from the nucleolar isolate) is electrophoresed, the promiment (nucleolar) peak is essentially absent, while the remainder of the pattern is similar to that obtained from whole nucleus. These data strongly suggest that the prominent iron-binding peak is largely, if not solely, a nucleolar component.

Nuclear iron appears to be either directly or indirectly linked to protein since trypsin treatment before electrophoresis markedly and concurrently increases the mobility of both iron- and lysine-labeled material (Fig. 7). The possibility that nuclear iron (except for the nucleolar fraction) is indirectly bound to protein through some nonprotein intermediate is suggested by the electrophoretic pattern obtained when the concentration of SDS in the gel is raised from 0.1% to 1.0% (Fig. 8). Under these conditions, migration of nucleolar iron is unchanged; however, the extranulceolar iron shows increased mobility and is spread over a more narrow range, thus effectively separating it from the nucleolar peak (compare Figs. 8 and 5). Also note that a single lysine peak coelectrophoreses with the nucleolar peak, indicating that in this organelle iron is bound to just one protein species. Essentially all of the nuclear iron counts remain acid-precipitable after electrophoresis on 1% SDS gels, indicating they are still bound to relatively large molecules. The increased concentration of SDS would not be expected to dissociate protein; this is emphasized by the similarity of lysine patterns in Figs. 8 and 5, and, therefore, it may be that iron is bound to its several nuclear protein species by way of a single type of macromolecule or a small number of macromolecules. We do not have data relating to their identity; however, insensitivity to DNase, RNase, and α- and β-amylase rule out several candidates.

While the binding sites discussed were assessed initially by labeling intact cells, we have observed that iron binds to these same sites *in vitro*. Thus when isotopic iron citrate is added to isolated nuclei or cytoplasm, the only acid-precipitable counts are those localized on the groups of macromolecules we have already pointed out. The bond is formed rapidly, irreversibly, and at 0°.

Although the significance of bound iron in HeLa cells is not fully clarified by these experiments, the subject would seem worthy of further attention, considering the apparent iron requirement for DNA synthesis.

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