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Research Articles

Availability of Ionic Iron from Iron Chelates

By WINTHROP E. LANGE, ALFRED G. BARNET†, and WILLIAM O. FOYE

Several commercial hematinic preparations have been tested for their ability to liberate ionic iron at different conditions of pH and in the presence of simulated gastric and intestinal fluids. A chromatographic method was employed in which both Fe(II) and Fe(III) ions were measured by means of absorbance determinations. It was concluded that chelated, or complexed, iron is carried through the gastrointestinal tract with less loss and lower toxicity than is ionic iron.

Some confusion exists regarding the requisites for the state of iron necessary for therapeutic use as a hematinic. Brading, *et al.* (1), for instance, have reported that more Fe(III) than Fe(II) was absorbed by rats fed inorganic Fe^{59}, and that the distribution in the tissues did not depend on either dose or valency of iron. Hartwig, *et al.* (2), also found that $Fe^{59}Cl_3$ was incorporated into new erythrocytes to a significantly greater degree than was Fe^{59}(II) citrate. Elsewhere it has been stated that only ferrous iron can be absorbed and that the ferric form must first be reduced before it can enter the gastrointestinal mucosa (3).

The advantages of chelated *vs.* inorganic iron for therapeutic use also appear to be in question. Franklin, *et al.* (4), claimed that chelation of iron minimized its toxicity and did not impair its hemopoietic response in humans. A difference in effectiveness of iron chelate preparations has been noted (5), however, which is evidently due to relative ease of liberation of ionic iron from iron chelates. Injectable ferric ammonium citrate, for instance, gave low hemoglobin levels and erythrocyte counts in weanling pigs in comparison with injectable iron-dextran, oral iron in

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paste form, and ferrous sulfate. Only the ferrous sulfate maintained normal blood values.

It is well known that ferric chelates show much more stability than ferrous in regard to liberation of ionic iron, since Fe(III) falls well above Fe(II) in the Mellor-Maley and Irving-Williams series. Also, in regard to oxidation-reduction potentials, it is known that when a ligand is anionic, the higher valence state of the metal is favored (6). Furthermore, ferric iron in many of its chelates is more difficult to reduce than inorganic ferric ion (6, 7).

The foregoing facts lead obviously to questions regarding the availability of iron in hematinic preparations where chelating agents are employed, the possibility, or necessity, of reduction of Fe(III) to Fe(II), and the competition of the administered ligand with cellular ligands where iron chelates of sufficient liposolubility to penetrate cells are present. In order to look into some of these problems, several commercial hematinic chelates have been subjected to various conditions under which ionic iron might be liberated. A comparison has been made with inorganic Fe(II) and Fe(III), and chelates containing both Fe(II) and Fe(III) were included.

The possibility also exists of iron exchange between the administered chelate and the heme pigments, without liberation of ionic iron, since the porphyrins have such an extreme affinity for iron (as yet no measurement of iron-porphyrin stability constants has been possible). In this connection, however, Rubin (8) has shown that chelating agents of the EDTA type were unable to remove iron from siderophilin, and siderophilin was likewise unable to remove iron from the tighter chelates.

EXPERIMENTAL

Separation of Fe(II) and Fe(III) Ions.—The method of Stevens (9) was used with some modification. A chromatogram holder consisting of four glass tubes fitted with corks into which horizontal glass rods were inserted and held in place by rubber bands was constructed to avoid contamination from metal racks or clips. The developing solution was a mixture of 40 parts of *n*-butanol, 25 parts of ethanol, 25 parts of glacial acetic acid, and 35 parts of distilled water. The solvent was allowed to equilibrate in the tank overnight before use. Whatman No. 1 chromatography paper was cut into strips 15 in. by 2 in., washed with 2 *N* hydrochloric acid for 30 minutes, and rinsed with distilled water until a pH of 6.5 to 7 was reached. The strips were allowed to dry in air.

Solutions of $FeSO_4 \cdot 7H_2O$ and $Fe_2(NH_4)_2(SO_4)_2 \cdot 24H_2O$ were prepared to contain a concentration of approximately 1 mcg./μl. The pH of the ferrous sulfate solution was buffered to 4, and the ferric ammonium sulfate solution to 2.5, using saturated sodium acetate solution and sulfuric acid. This was done to minimize reduction and hydroxide formation. The solutions were spotted on the paper in 1 to 5 μl. amounts by means of a micropipet under a stream of warm air. The ferrous solution was spotted first and allowed to dry; the ferric solution was superimposed on the ferrous spot and allowed to dry. The strips were placed in contact with the developing solution and allowed to develop for 3.5 to 4 hours. The strips were removed, allowed to dry in air, and then exposed to water vapor containing a small amount of 8-hydroxyquinoline (oxine) from the side-arm of a distilling flask. The presence of Fe(II) and Fe(III) ions was indicated by green-black spots. The R_f value for Fe(III) was found to be 0.57, and that for Fe(II) was 0.24. The temperature was kept at 23–25°.

Preparation of a Standard Curve.—Exact quantities of 1 to 5 μl. of the standard solutions (containing 0.5 to 1.5 mcg. per μl.) were spotted on strips of Whatman No. 1 chromatography paper and placed in contact with the developing solution for 4 hours. The strips were removed and dried in air, and the spots were made visible by exposure to water vapor containing oxine. The strips were allowed to dry, and the maximum and minimum absorbances were determined using a Welsh Densichron densitometer, model 3853D, with a 3-mm. aperture. The minimum absorbance was that reading obtained from the paper itself. The average absorbance was determined by adding the maximum and minimum absorbances, dividing by two, and subtracting the paper blank. The areas of the spots were determined by tracing on graph paper and counting the squares, or by use of a planimeter.

Six to ten chromatograms were prepared for each known concentration, unsatisfactory results being discarded. An average of the results of the runs for each concentration was taken and the area multiplied by the average absorbance was plotted *vs.* the concentration.

Quantitative Determinations of Iron.—Each product or compound was analyzed under five different conditions: (*a*) untreated, (*b*) after treatment with stimulated gastric fluid U.S.P. XVI, (*c*) after treatment with simulated intestinal fluid U.S.P. XVI, (*d*) after treatment with 5 ml. of concentrated hydrochloric acid, and (*e*) after treatment with a solution of pH 7.5.

(*a*) *Untreated.*—An equivalent of the compound or product was diluted to 100 ml. with distilled water so that each microliter contained approximately 1 mcg. of iron. The paper strips were spotted in 1 to 5 μl. amounts and developed as described above. The average absorbances and areas of the spots were determined as described, and the concentration of iron read from the standard curve.

(*b*) *Treatment with Simulated Gastric Fluid.*—An equivalent of the compound or product was diluted to 100 ml. with simulated gastric fluid so that each microliter contained approximately 1 mcg. of iron. The solution was placed in a constant temperature bath for 1.5–2 hours at 37° with occasional agitation. The paper strips were spotted with 1 to 5 μl. amounts, and concentrations were determined.

(*c*) *Treatment with Simulated Intestinal Fluid.*—This procedure was carried out in the same manner as in (*b*).

(*d*) *Treatment with Concentrated Hydrochloric Acid.*—An amount of the compound or product was diluted with 5 ml. of hydrochloric acid and enough distilled water to make 100 ml. so that each microliter contained approximately 1 mcg. of iron. The solution was allowed to stand for 4 hours with occasional shaking, and paper strips were spotted with 1 to 5 µl. amounts. The remainder of the procedure was as described above.

(*e*) *Treatment with a Solution of pH 7.5.*—An amount of the product or compound was diluted to 100 ml. with an aqueous solution of pH 7.5, obtained with phosphate buffer, such that each microliter contained approximately 1 mcg. of iron. The solution was placed in a constant temperature bath at 37° for 1.5–2 hours with occasional agitation. The strips were spotted with 1 to 5 µl. amounts, and the concentrations of iron determined in the customary manner.

RESULTS AND DISCUSSION

In order to make comparisons of the amount of ionic iron liberated from commercial hematinic preparations under different conditions of acidity, a convenient assay procedure was required in which other components of the preparations, such as syrups, vitamins, coloring agents, calcium salts, phosphates, and the organic ligands, do not interfere. A chromatographic separation of Fe(II) and Fe(III) ions was carried out essentially as described by Stevens (9) except that ascending rather than descending development was employed. Quantitative estimation of the ionic iron liberated from the various commercial preparations and the inorganic iron salts selected as control compounds was based on techniques previously described by Block, Durrum, and Zweig (10). The method consisted primarily of measurement of area and absorbance of chromatographic spots of liberated ionic iron in the presence of oxine. A standard curve was drawn, plotting average absorbance times area *vs.* concentration and found to follow the Lambert-Beer law. A considerable number of volumes and concentrations were employed before optimum experimental conditions were determined.

Some difficulty was encountered in determining the areas of the spots, largely because of the contaminating ingredients present in the commercial preparations. Not only area, but also regularity and R_f value were affected, but employment of a sufficient number of determinations generally gave satisfactory average values. It was possible to determine ionic iron in amounts less than 0.5 mcg. per µl. by this procedure.

The relative amounts of ionic iron liberated under varying conditions of acidity: (*a*) without prior treatment, or after treatment with (*b*) simulated gastric fluid, (*c*) simulated intestinal fluid, (*d*) concentrated hydrochloric acid, and (*e*) aqueous solution of pH 7.5 were determined (see Table I). Treatment with concentrated hydrochloric acid was designed to liberate all of the iron present, and treatment with simulated gastric and intestinal fluids was carried out to determine effect of pH, pepsin, and pancreatin on the state of oxidation and release of ionic iron. Solutions of pH 7.5 were designed to show any effects due to pancreatin other than a pH effect. Treatment with simulated intestinal fluid and solution of pH 7.5 generally caused extreme tailing of the chromatographic spots, so these results are not included in Table I. The R_f value of the iron also was not reached in the former case, evidently due to combination of iron with pancreatin.

No significant change in oxidation state of iron was noted in any of the products under the conditions of acidity employed. Peptonized iron, ferrous gluconate, ferrous glycinate, and iron choline citrate all remained in the ferrous state, whereas the iron-carbohydrate complexes remained in the ferric state. The ferric complexes, as might be predicted, were the more stable and, in one instance, liberated no ionic iron even after treatment with simulated gastric fluid (pH 1.2). With the exception of the ferric-carbohydrate complexes, simulated gastric fluid liberated essentially all of the iron from the complexes studied.

It may be concluded that the more tightly bound ferric complexes are more successful in carrying iron through the gastrointestinal tract to the areas of absorption without loss of iron to pancreatin. Toxicity due to liberation of ionic iron should also be less with the more tightly bound complexes, and

Table I.—Assay Results of Various Iron Preparations

Product	Iron Present As	Oxidation State Found	Amount Recovered, %[a]		
			Hydrochloric Acid[b]	Untreated	Simulated Gastric Fluid
Livitamin with iron	Peptonized iron	+ +	97.00	59.86	108.47
Fergon compound elixir	Ferrous gluconate	+ +	90.00	76.99	90.85
Ferronord	Ferrous sulfate glycine complex	+ +	101.25	99.00	102.5
Imferon	Iron dextran complex	+ + +	92.40	0.0	25.80
Ferrolip	Iron choline citrate complex	+ +	96.00	69.50	94.67
Jefron elixir	Iron carbohydrate complex	+ + +	108.0	0.0	0.0
Saccharated iron oxide	Iron carbohydrate complex	+ + +	74.27	0.0	22.92
Ferrous sulfate[c]	...	+ +	99.63	100.0	97.64
Ferric ammonium sulfate	...	+ + +	99.51	100.0	98.06

[a] The percentages given are based upon the theoretical values given by the manufacturer. Iron concentrations could not be determined in alkaline media of pH 7.5 or simulated intestinal fluid. [b] The percentages given are the average values for a minimum of six determinations. [c] The percentages given are based upon the results of the findings of potassium permanganate assay.

there is already evidence to indicate that chelated iron is less toxic than ionic iron (4). In regard to subsequent distribution and utilization of iron, it may well be the case that chelation is a necessary requirement, since transport of ionic iron, *per se*, through cellular membranes is questionable. Release of iron from administered chelates to form hemoglobin should present no obstacle because of the known extreme affinity of porphyrins to chelate iron (11).

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Composition of Gum Turpentines of Pines

Pinus nelsonii and *Pinus occidentalis*

By NICOLAS T. MIROV, EUGENE ZAVARIN†, and JOSEPH G. BICHO†

It was found that the turpentine of *Pinus nelsonii* contained 49.60% *l*-α-pinene, 41.50% *l*-β-pinene, 1.02 dipentene, 0.24% camphene, and 0.01% *n*-heptane. The remainder contained 3.74% of oxygenated substances derived mainly from terpenes (including probably some oxygenated sesquiterpenes); about 0.02% of unidentified, low-boiling materials, pot residue, and losses. The possible occurrence in *P. nelsonii* turpentine of the oxygenated materials should be rechecked, using freshly collected material. The identification of camphene and *n*-heptane must be considered as tentative, only. The turpentine of *Pinus occidentalis* contained 63.8% *d*-, *dl*-α-pinene, 22.2% *l*-β-pinene, 7.7% *d*-Δ³-carene, 1.8% methyl chavicol, 1.1% dipentene, 0.5 % *d*-longifolene, 0.3% linalool, 0.2% camphene, and 0.1% *n*-heptane, with the remaining 2.3% accounted for by unidentified terpenes, pot residue, and losses. The identification of the last four constituents must be considered only tentative.

PINUS NELSONII Shaw is a Mexican piñon or nut pine, growing in a few restricted localities in the desert ranges of Mexican states Tamaulipas, Nuevo Leon, Coahuila, and San Luis Potosí. It is a small bushy tree with long pliant branches, sparse gray-green foliage, and gray bark. Its three cohering needles give the impression of a single, slender needle. A sample of the oleoresin of this pine was received from Aramberri, Nuevo Leon, Mexico. The trees from which the oleoresin was procured grew at an elevation of about 7000 ft. in Valle Hermoso, Miquihuana, Tamaulipas. Herbarium specimens accompanied the oleoresin sample.

Pinus occidentalis Swartz is a pine of the Caribbean area. It grows in eastern Cuba and on the Island of Hispañola, both in the Dominican Republic and in Haiti. It is closely related to *Pinus caribaea* and also to other pines of Mexico and of the southeastern United States. A generous sample of oleoresin of this pine was collected in August of 1960 at Mare Range section of the Pine Forest, Haiti, courtesy of SHADA.

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DISCUSSION AND EXPERIMENTAL

Pinus Nelsonii

The turpentine was distilled under reduced pressure. Toward the end of the operation, the pressure was reduced to 2 mm. and the temperature reached 215°. At the end, small quanitities of distillate solidified in the condenser. The pot residue was hard and brittle, showing that all volatile components, including sesquiterpenes and their derivatives, were removed from the oleoresin. Yield of turpentine was 27.8%; its physical characteristics were: density, d_4^{25}, 0.8557; index of refraction, n_D^{25}, 1.4711; and specific rotation, $[\alpha]_{578}^{23}$ −38.5. This was reported by Mirov (1).

A charge of 309.7 Gm. of *P. nelsonii* turpentine was distilled through a 90 cm. long, 12 mm. inside