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# REDISSOLVABLE FERRIC–D-FRUCTOSE AND FERRIC–D-GLUCOSE–D-FRUCTOSE COMPLEXES

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ABSTRACT

Reinvestigation of the chelation of iron by D-fructose and D-glucose led to definable conditions for the preparation of ferric–D-fructose and ferric–D-glucose–D-fructose complexes that could be isolated and redissolved to give neutral solutions. The complexes isolated had defined and reproducible compositions. The behaviour of complexes prepared with $^{14}C$-labelled carbohydrates revealed no interconversion of D-fructose and D-glucose in ferric–D-glucose–D-fructose complexes.

## INTRODUCTION

Ferric iron will not remain in solution at a pH greater than ~2, whereas precipitation of ferrous iron normally occurs above ~3.5. Iron compounds that are soluble and stable over a wide pH-range are of considerable importance in the treatment of anaemia. It is generally accepted that iron is absorbed by the body in any region from the stomach distal to the intestines, though this process occurs mainly in the duodenum[1], where the environment is neutral or slightly alkaline. Polyols and carbohydrates may compete with hydroxide ions as ligands for the metal cation, thereby preventing hydrolysis and effectively solubilising the iron. Thus, in the formulation of iron–carbohydrate complexes, a balance between pH and the concentrations of the metal ion and the carbohydrate must be attained so as to favour chelation and solubilisation of the metal by the carbohydrate, as opposed to hydrolysis by hydroxide ions. The object of the work described here was to provide a convenient route to reproducible preparations of iron–D-fructose complexes.

## RESULTS AND DISCUSSION

Saltman *et al.*[2] studied the effects of pH and both the relative and absolute concentrations of reagents on the solubilisation of iron by D-fructose. Three ranges of concentration were defined, where (*1*) soluble complexes were invariably obtained, (*2*) complexes were "unstable" in the pH range 5–9, and (*3*) complexes were "unstable" at all pH values. The precise meanings of "soluble" and "unstable" in this

context were not stated. Isolated samples of ferric–D-fructose complexes, prepared by the method of Saltman, could not generally be redissolved. Furthermore, Saltman's investigations only included iron concentrations up to 0.6M.

From preliminary experiments, it became apparent that the ability of D-glucose to sequester iron and form redissolvable complexes was far inferior to that of D-fructose. Although the isolated complexes eventually redissolved, the ferric–D-glucose complexes were far less water-soluble than the ferric–D-fructose complexes and therefore were not studied further. However, attempts were made to substitute 50% of the D-fructose with D-glucose, in the hope that the added D-glucose might satisfy the excess of carbohydrate required for successful chelation. Such a mixture is readily available as invert sugar and thus would provide a convenient commercial source of D-fructose for complex formation. The ferric–D-glucose–D-fructose and ferric–D-fructose complexes were therefore studied in parallel.

The pH and concentration were interrelated in their influence on the formation of the complexes. Nevertheless, certain generalisations could be made about the formation of redissolvable, stable samples of the complexes. Complexes prepared at pH $<6.0$ were insoluble after isolation, although no precipitation of iron was detected during their preparation. Precipitation often occurred in a critical pH range between $\sim 6.0$ and 8.0. Solutions of either complex could be prepared in this pH range, provided a sufficient excess of carbohydrate was employed at a suitably high concentration. However, such samples were insoluble after isolation, with one exception. This was a ferric–D-fructose complex prepared at pH 8.0, which is on the upper limit of the critical range.

The properties of these two groups of preparations are in apparant contradiction with those found by Saltman[2], who reported the formation of soluble, stable complexes at concentrations similar to those employed here. However, Saltman did not state whether the complexes were isolated, or whether they were generated solely in the reaction mixture.

D-Fructose has a sequestering ability slightly superior to that of D-fructose–D-glucose mixtures, although the latter are still very much more effective than D-glucose.

Complexes prepared above pH 8.0 were soluble after isolation. Precipitation, though frequently only transient, often occurred when passing through the critical pH range, but redissolution always occurred, provided that the concentrations of reagents were controlled. By using a molar ratio of carbohydrate to iron of 16:1 or 20:1, this precipitation was minimised, and an increase in the absolute concentrations eliminated precipitation. It was also noted that the minimum absolute concentration of D-fructose required to prevent precipitation was less than for D-glucose–D-fructose mixtures.

When applied to solutions of high absolute concentrations ($\sim 3$M carbohydrate) and low ratios of carbohydrate to iron (2:1 or 3:1), the procedure used above gave rise to heavy precipitation of iron. In order to improve the yields of products with respect to carbohydrate in particular, attempts were made to prepare samples of both complexes by adding a solution of ferric chloride to a solution of carbohydrate maintained at an alkaline pH by the simultaneous addition of alkali. In such a system, the iron is,

in effect, continually chelated in the reaction solution carbohydrate, and the concentration of uncomplexed iron is low and in the presence of a large excess of carbohydrate. This method allowed iron–carbohydrate complexes to be prepared from solutions of high absolute concentrations and low molar ratios of carbohydrate to iron, which would not normally have given soluble chelates. The yields of complexes were greatly improved with respect to carbohydrate, and precipitation was totally eliminated. Attempts to prepare the complexes directly at pH 7.0 failed. However, utilising the observed hysteresis effects[2], solutions of complexes, once prepared, could be readily adjusted to a neutral pH without precipitation. After isolation, the samples were water-soluble, at concentrations up to 25%, to give solutions of neutral pH which were stable at 100°. Moreover, the samples were of reproducible composition. The elemental analyses (Table I) indicated that the complexes comprised carbohydrate, iron, and sodium in the molar ratios ~2:2:1, in accordance with previous findings[2].

TABLE I

ANALYSES[a] OF IRON–CARBOHYDRATE COMPLEXES

| *Preparation No.* | *Ferric–D-fructose* | | *Ferric–D-glucose–D-fructose* | |
|---|---|---|---|---|
| | *1* | *2* | *3* | *4* |
| C | 23.6 | 24.7 | 23.6 | 24.1 |
| H | 4.5 | 4.8 | 4.2 | 4.6 |
| Fe | 18.9 | 18.8 | 19.3 | 19.5 |
| Na | 3.4 | 3.6 | 3.7 | 3.2 |
| Cl | 4.3 | 4.4 | 4.6 | 4.3 |
| D-Fructose | 70.2 | n.d.[c] | 44.1 | n.d. |
| D-Glucose | 0.0 | n.d. | 12.6 | n.d. |
| $H_2O$[b] | 8.1 | 7.7 | 12.6 | 12.1 |

[a]Values expressed as % w/w. [b]Loss in weight on drying at 108° *in vacuo* over $P_2O_5$. [c]n.d. = Not determined.

Though elemental analyses indicate the amounts of iron and carbohydrate, *inter alia*, present in the complexes, they do not reveal the nature of the complexes or the products of dissociation. Direct determination of iron and carbohydrate by spectrophotometric procedures could not be employed, since this depends on the total dissociation of one complex to form a second. A means of dissociating the complexes and separating the iron from carbohydrate was therefore required. It was desirable that any operations carried out on the samples were performed at a neutral pH in order to minimise rearrangements. Analyses both of ferric–D-fructose and ferric–D-glucose–D-fructose complexes gave reproducible results (Table I). A full account of the compositions of the complexes is prevented by the lack of a procedure for the reliable determination of water of hydration which is certain to be associated with the complexes, since the binding constants involved between a coordinated water molecule and a metal cation vary as to the nature of the other ligands present. For comparison, the loss in weight on drying is included.

As the complexes were generated in an alkaline medium, the possibility of some alkaline degradation of the carbohydrate could not be excluded. Fractionation of the dissociated complexes by anion-exchange chromatography afforded a variety of acidic products, present only in small amounts (0.15% of complex). Analysis of the complexes after storage for six months indicated an increase in these products to 2.0%. Dissociation and fractionation of the $^{14}C$-labelled complexes by anion-exchange chromatography and radioassay of the fractions confirmed that all acidic degradation products had been detected fluorimetrically.

Ferrous iron in the complexes was determined by the 2,2′-bipyridyl assay after removal of the ferric iron by solvent extraction with acetylacetone at pH 3.0. Direct application of the assay was ineffective since the ferric iron was readily reducible under the conditions used. Values for ferrous iron in a ferric–D-fructose complex of 0.37 and 0.34% were recorded.

In order to verify the validity of the method of dissociation of the complexes and subsequent analyses, samples of both types of complex were prepared incorporating $^{14}C$-labelled carbohydrates, *viz.*, ferric–D-fructose-*1*-$^{14}C$, ferric–D-glucose–D-fructose-*1*-$^{14}C$, and ferric–D-glucose-*1*-$^{14}C$–D-fructose. Determination of the activities of the three samples by thin-film planchet counting gave the carbohydrate contents shown in Table II, together with those obtained by dissociation and fractionation of the complexes. The two methods of assay are in good agreement.

TABLE II

ANALYSIS[a] OF RADIOACTIVELY LABELLED IRON–CARBOHYDRATE COMPLEXES

| *Sample* | *Radioassay* | | *Colorimetric assay* | |
|---|---|---|---|---|
| | *Fructose* | *Glucose* | *Fructose* | *Glucose* |
| Ferric–D-fructose-*1*-$^{14}C$ | 63.8 | 0.0 | 59.2 | 0.0 |
| Ferric–D-glucose-D-fructose-*1*-$^{14}C$ | 47.9 | 0.0 | 45.6 | 8.9 |
| Ferric–D-glucose-*1*-$^{14}C$–D-fructose | 0.0 | 9.8 | 46.5 | 9.1 |

[a]Values expressed as % w/w.

Radioassay, by liquid scintillation counting, of the fractions collected by column chromatography of the dissociated complexes containing D-fructose-*1*-$^{14}C$ gave no labelled D-glucose; similarly, the ferric–D-glucose–D-fructose sample incorporating D-glucose-*1*-$^{14}C$ gave no labelled D-fructose. Both ferric–D-glucose–D-fructose samples afforded D-glucose and D-fructose. D-Mannose was not detected (colorimetrically or by radioassay) in any fractionation. Thus, the carbohydrate used in complex formation was always recovered in the same form.

These results preclude the suggestion made by Saltman[2] that, in the ferric–D-fructose complexes, the carbohydrate was present as an ene-diol, which should give a mixture of D-fructose, D-glucose, and D-mannose on dissociation. Although the rate of

enolisation of D-fructose is greater than that of D-glucose, this factor alone is not sufficient to explain the differing abilities of the two sugars to sequester iron, particularly since D-glucitol (which cannot enolise) has a high ability to complex iron[2]. It is more probable that the complexes are formed by a mechanism involving alcoholates of the carbohydrate in a manner similar to that proposed by Traube[3], although the high pKa values of carbohydrates would not explain the observations that ferric–D-fructose complexes are formed in weakly acidic environments. Equally possible is a concerted mechanism in which direct exchange of water and hydroxyl (*i.e.*, carbohydrate) ligands is involved. Loss of a proton (hence Saltman's observations) to form an ionic salt might stabilise the carbohydrate ligands towards hydrolysis. Thus, the abilities of carbohydrates to chelate iron may depend on certain structural and stereochemical aspects in one of their configurations. Several stereochemically favourable structures for D-fructose in ferric–D-fructose complexes can be envisaged, wherein D-fructose acts, for example, as a tridentate ligand for iron *via* O-1,O-2,O-3 of β-D-fructopyranose, or O-1,O-6,O-5 of α-D-fructofuranose, or as a bidentate ligand for iron *via* the *cis* pairs of oxygen atoms O-2,O-3 and O-4,O-5 of β-D-fructopyranose.

Attempts to prepare ferrous complexes in the presence of air and at an alkaline pH gave dark-green solutions of complexes; though possessing excellent water-solubilities, the complexes were rapidly oxidised to the ferric state. In an atmosphere of nitrogen, heavy precipitation of iron occurred, and redissolution was not effected during 24 hours. The instability of the ferrous complexes towards oxidation bears a marked similarity to the findings of Cox and King[4] with respect to iron–dextran complexes.

Thus D-fructose or D-fructose–D-glucose mixtures have an appreciable ability to solubilise iron, and the complexes so formed are suitable for physiological studies. The improved method of preparation requires a minimum amount of carbohydrate, and the complexes have defined and reproducible composition.

## EXPERIMENTAL

*Preparation of iron–carbohydrate complexes.* — (*a*) Solutions of carbohydrate (0.32–3.2M, 1 vol.) and of ferric chloride ($FeCl_3 \cdot 6H_2O$, 0.02–1.5M, 1 vol.) or ferrous sulphate ($FeSO_4 \cdot 7H_2O$, 0.32–1M, 1 vol.) were mixed and appropriately adjusted to pH 5.0–11.5 with 0.1–8M sodium hydroxide. Water-soluble complexes were precipitated by the addition of ethanol (4 vol.), and washed with ethanol (80%, ~200 ml). The precipitated complexes were redissolved in distilled water and isolated by freeze-drying.

(*b*) Solutions of ferric chloride ($FeCl_3 \cdot 6H_2O$, 1–1.07M, 500 ml) and sodium hydroxide (8M) were added simultaneously and dropwise to solutions of carbohydrate (3.0–3.2M) so that the pH was maintained at either 8.5 or 9.0. No precipitation occurred. The complexes were precipitated, collected and washed as described above, and redissolved in distilled water (1500 ml). After the solutions had been adjusted to pH 7.0–7.5, the complexes were isolated by freeze-drying.

*Elemental analyses.* — Iron was determined by gravimetric analysis[5], chloride by acid decomposition[6,7], and sodium by flame photometry.

*Anion-exchange chromatography.* — (*a*) Neutral sugars were fractionated by chromatography on a column (75 × 0.6 cm) of Dowex-AG1 X8 (200–400 mesh, borate form) with a borate buffer (16mM $Na_2B_4O_7$, 0.2M $H_3BO_3$). The eluate was monitored by the automated, spectrophotometric, cysteine–sulphuric acid assay[8].

(*b*) Acidic carbohydrate materials were fractionated on a column (140 × 0.6 cm) of Dowex-AG1 x8 (200–400 mesh; acetate form) by elution with 0.5M acetic acid. The column eluate was monitored by the automated, fluorimetric, formaldehyde assay[9].

*Thin-film planchet counting.* — Solutions of the samples to be counted were transferred to flat, aluminium planchets (2-cm diameter) and dried under a lamp. The samples were mounted in a lead castle close to an end-window Geiger–Muller tube linked to a scalar. The mean of six 5-min counts was taken as a measure of the activity of the sample. From the count-rates of samples of D-glucose-*1*-$^{14}C$ of known activities (0–30 $\mu$Ci), an efficiency of 4.6% was ascertained for the equipment and no evidence of self-absorption phenomena was observed.

*Liquid scintillation counting.* — Aliquots (1 ml) of aqueous solutions of $^{14}C$-labelled carbohydrates were emulsified with the phosphor (10 ml) in "low potassium" counting phials (Hewlett Packard Ltd.). The phosphor was prepared by dissolving 2-(4′-*tert*-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole (Butyl-PBD, 15 g) in scintillation-grade toluene (660 ml). Triton X-100 emulsifying agent (330 ml) was added and the solution thoroughly mixed. Measurements were recorded on an Intertechnique ABAC SL40 Liquid Scintillation Counter.

*Dissociation of iron–carbohydrate complexes by solvent extraction.* — Solutions of iron–carbohydrate complexes (0.02–2.0 g) in water (10–25 ml) were shaken with pentane-2,4-dione (acetylacetone) (redistilled, 1-5 ml) for 15 min. The red acetylacetonato–iron(III) complex was extracted into toluene, using a continuous liquid–liquid extraction apparatus, until 10 minutes after there was no further colour transfer to the organic layer. It was important to maintain the extraction vessel at 0°.

*Determination of ferrous iron in iron–carbohydrate complexes.* — A solution of the complex (~30 mg) in deionised, deaerated water (15 ml) was adjusted to pH 3.0 with 0.5M hydrochloric acid and diluted to 25 ml. An aliquot (10 ml) of this solution was dissociated and the ferric iron removed by solvent extraction as above. The residual aqueous layer was shaken with 2,2′-bipyridyl reagent (5 ml), prepared by dissolving 2,2′-bipyridyl (250 mg) in ethanol (5 ml) and diluting to 100 ml with deionised water. The mixture was shaken for 10 min and a solution of sodium lauryl sulphate (5% w/v, 5 ml) added. After shaking for a further 5 min, the red ferrous–bipyridyl complex was extracted into 2-methylpentan-1-ol (1 × 15 ml, 5 × 5 ml). The extracts were combined and diluted to 50 ml and the absorbance was determined at 510 nm.

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