THE DEGRADATION OF HYALURONIC ACID BY FERROUS IONS*

J. F. KENNEDY AND H. CHO TUN
Department of Chemistry, University of Birmingham, Birmingham B15 2TT (Great Britain)
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ABSTRACT

A study of the degradation of hyaluronic acid by ferrous ions under atmospheres of nitrogen, air, and oxygen has shown the necessity of oxygen for reaction. That degradation occurred was demonstrated by the decrease in specific viscosity of the solution, an increase in the number of acid groups, and the formation of residues that yielded β-formylpyruvic acid on periodate oxidation. Digestion of the ferrous-degraded mixture with hyaluronidase (E. C. 3.2.1.35), β-D-acetamidodeoxyglucosidase (3.2.1.30) and β-D-glucuronidase (3.2.1.31), followed by ion-exchange chromatography and gel filtration, yielded an unsaturated component which behaved as 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose. Furthermore, the degradation by ferrous ions rendered the hyaluronic acid incompletely hydrolysable by the three enzymes.

INTRODUCTION

Hyaluronic acid, a polysaccharide having a repeating unit of 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-D-glucose, is degraded by various autoxidants such as ferrous, cuprous, and stannous ions, L-ascorbic acid, D-isoascorbic acid, and D-dehydroisoascorbic acid, and by X-rays, ultraviolet radiation, and ultrasonic waves[1–8]. The presence of oxygen has been reported to be essential for the degradation with autoxidants[9], and the mechanism of degradation is probably similar to that of free-radical degradation by radiation[6,10,11]. This autoxidation of hyaluronic acid has been described by Pigman[9] as an oxidative–reductive depolymerization reaction. Of all the autoxidants, ferrous ion was the most effective[7,9,12], giving maximal degradation[13] in the pH range 4–7, and phosphate ions accelerated the degradation[9,14].

EXPERIMENTAL AND RESULTS

Assessment of untreated, commercial preparations of hyaluronic acid and its potassium salt. — Two batches of potassium hyaluronate (*A* and *B*, Koch–Light Laboratories Ltd.; human, umbilical cords) and a batch of hyaluronic acid (*C*, Nutritional Biochemical Corporation; bovine vitreous humour) were dissolved in water and assayed by the Warren[15] technique, using neuraminic acid as the standard. Spectrophotometric measurements were made at 549 nm, and the absorption spectra

*Dedicated to Professor M. Stacey, C.B.E., F.R.S., in honour of his 65th birthday.

of the chromophores produced in each case were determined. Batches *A* and *B* contained small amounts of Warren-positive material equivalent to 1.36 and 1.16 μmoles, respectively, of β-formylpyruvic acid/g of potassium hyaluronate, whereas batch *C* contained less than the equivalent of 0.56 μmole of β-formylpyruvic acid/g of hyaluronic acid. The samples showed the same absorption spectra as the standard, having absorption maxima at 549 nm.

Analytical-scale degradation of hyaluronic acid by ferrous ions and initial assay of the products. — Hyaluronic acid solution (250 μg/ml) in 5 mM sodium phosphate buffer at pH 5.0 (4.0 ml) was mixed, in an Ostwald Viscometer maintained at 37° in a constant temperature bath, with 9.7 mM ferrous sulphate in 5 mM sodium phosphate buffer (pH 5.0, 150 μl), both solutions being pre-equilibrated in the bath. The specific viscosity of the mixture was determined at intervals during the degradation and, simultaneously, aliquots were taken from the reaction mixture and assayed by the Morgan–Elson[16] spectrophotometric assay for reducing 2-acetamido-2-deoxy-D-glucopyranose residues. The specific viscosity dropped to 2.9% of the zero-time value at 210 min, after which it remained constant. No reducing 2-acetamido-2-deoxy-D-glucose was detected at any stage during the degradation; appropriate controls of ferrous ions added to standard solutions of 2-acetamido-2-deoxy-D-glucose showed no deviation in colour yield from the standards themselves. Warren assay was carried out on an aliquot of the final, degraded product after degradation had proceeded for 24 h, using *N*-acetylneuraminic acid as a standard. The chromophore produced was equivalent to 44.9 μmoles (corrected) of β-formylpyruvic acid/g of hyaluronic acid and had the same absorption spectrum as the standard. Appropriate controls showed that the presence of ferrous ions at the concentration used decreased the colour intensity derived from *N*-acetylneuraminic acid to 64% of the value obtained in their absence. Further investigation of the effect of this ferrous concentration on the colour yield in the Warren assay using barium isosaccharinate showed decreases in the range of 74–58%, according to the concentration of the carbohydrate.

Assessment of the increase in alkali-titratable groups during ferrous degradation of the hyaluronic acid. — Hyaluronic acid (48.6 mg) was dissolved in 5 mM sodium phosphate buffer (pH 5.0, 10.0 ml) and ferrous sulphate ($FeSO_4 \cdot 7H_2O$, 21 mg) was added. Control solutions of hyaluronic acid (51.4 mg) in phosphate buffer (10 ml), ferrous sulphate (21 mg) in phosphate buffer (10 ml), and phosphate buffer only (10 ml) were also maintained at 37° for 24 h. All solutions were protected from atmospheric carbon dioxide. The solutions were then titrated with 25 mM sodium hydroxide, using a pH meter. From the titration curves, it was calculated that, during the degradation, 488 μequivalents of alkali-titratable groups were formed/g of hyaluronic acid.

Investigation of oxygen dependence of the ferrous degradation of hyaluronic acid. — *(a) Ferrous treatment under nitrogen, air, and oxygen.* Three solutions of hyaluronic acid (*C*, 140 μg/ml) in 5 mM sodium phosphate buffer (pH 5.0, 10 ml each) and three solutions of 4.5 mM ferrous sulphate in 5 mM phosphate buffer (pH 5.0, 1.0 ml) were prepared. In the first case, oxygen-free nitrogen, further purified by passage through

saturated, aqueous barium hydroxide, 20% w/v pyrogallol in 6M sodium hydroxide, conc. sulphuric acid, and finally calcium chloride, was passed for 15 min through the hyaluronic acid and ferrous solutions which were then mixed under nitrogen. Any reaction was allowed to proceed at 37° for 24 h under nitrogen at atmospheric pressure. In the second case, solutions were mixed in the presence of air, and degradation was allowed to proceed at 37° for 24 h in the presence of air at atmospheric pressure. In the third case, oxygen, purified by passage through saturated, aqueous barium hydroxide, conc. sulphuric acid, and calcium chloride, was passed for 15 min through the two solutions which were then mixed under oxygen; reaction was allowed to proceed as before. Warren assays were carried out on aliquots of the three ferrous-treated solutions. Degradation under nitrogen did not yield any Warren-positive products, whereas degradation under air and under oxygen yielded the equivalents of 0.82 and 3.26 mmoles, respectively, of β-formylpyruvic acid per mole of hyaluronic acid repeating-unit, the absorption maximum of the chromophores being 549 nm.

(*b*) *Assessment of products by enzymic degradation and fractionation.* The three ferrous-treated hyaluronic acid samples were incubated under their respective atmospheres at 37° for 24 h after addition of testicular hyaluronidase (Sigma Chemical Company, 390 i.u./mg, 1 mg). β-D-Glucuronidase (Koch–Light Laboratories, 2,000 Fishman units/mg, 6 mg) and β-D-acetamidodeoxyglucosidase (6 mg) were then added; the latter enzyme was prepared as described by Barker *et al.*[17], the fraction corresponding to precipitation at 30–40% saturated ammonium sulphate being selected. Incubation was continued for 48 h at 37°, and the three solutions were immediately deep-frozen and then freeze-dried. The residues were redissolved in water (1 ml) and were chromatographed on separate columns (22.7 × 0.57 cm) of Dowex AG-1 resin (acetate form, x8, 200–400 mesh). The columns were eluted with a gradient of 0–0.3M ammonium acetate at a flow rate of 0.60 ml/min. The eluates were assayed by the automated barbituric acid[18] assay, using Technicon modular equipment (Fig. 1). This assay is very similar to the Warren assay, the difference being the use of barbituric acid in place of 2-thiobarbituric acid; the chromophores produced with malonaldehyde and β-formylpyruvic acid have λ_{max} at 485 and 506 nm instead of 532

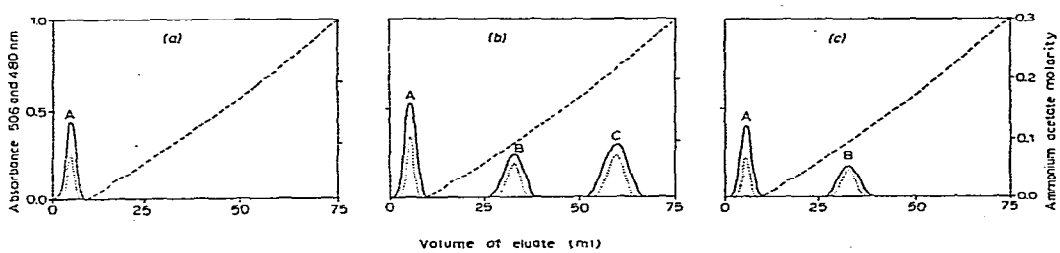

Fig. 1. Fractionation (Dowex AG-1 x8) of hyaluronic acid after ferrous and multi-enzyme treatment under (*a*) nitrogen, (*b*) air, and (*c*) oxygen; (———) and (·····), automated bartiburic acid assay, (506 nm) and (480 nm), respectively; (– – –), ammonium acetate gradient.

and 549 nm, repectively. The absorbances were actually measured by using 480 and 505 nm filters in the colorimeters.

Preparative scale, ferrous degradation of hyaluronic acid under air and oxygen. — (*a*) *Degradation, enzymic treatment, and ion-exchange fractionation of the products.* Degradations of hyaluronic acid (*C*, 180 mg) were carried out by the above method, with atmospheres of air and oxygen. Enzymic degradation was carried out as before, and the final products were freeze-dried and loaded on to separate columns (96 × 1.1 cm) of Dowex AG-1 resin (acetate form, x8, 200–400 mesh), and the columns eluted with a gradient of 0–0.3M ammonium acetate at a flow rate of 2.90 ml/min. Fractions (10 ml) were collected automatically, and appropriate aliquots were scanned by the carbazole[19], Morgan–Elson, and Warren assays (Fig. 2).

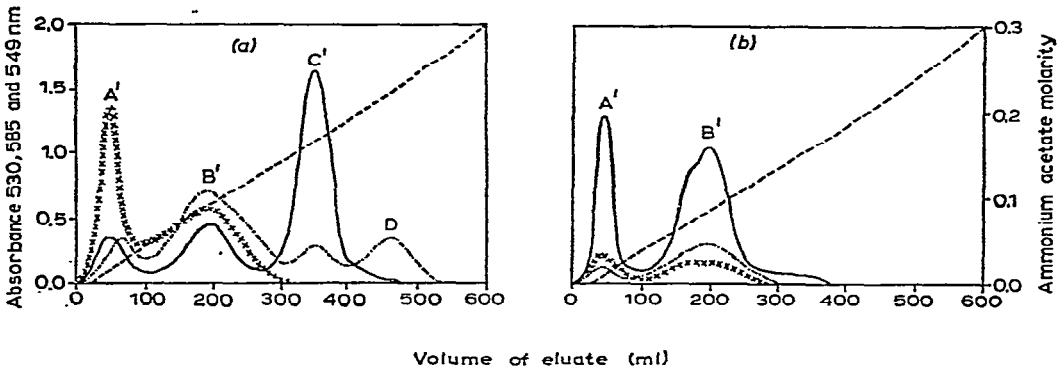

Fig. 2. Preparative scale fractionation (Dowex AG-1 x8) of hyaluronic acid after ferrous ion and multi-enzyme treatment under (*a*) air and (*b*) oxygen; (———), carbazole assay; (× × × ×), Morgan–Elson assay; (–·–·–), Warren assay; (– – –), ammonium acetate gradient.

(*b*) *Gel-filtration chromatography of selected fractions.* Fractions corresponding to peaks B′ in the case of degradations under air and oxygen were combined and freeze-dried. The products were dissolved in water (5 ml) and the ammonium ions removed by passing the solutions through columns (65 × 1.4 cm) of Dowex 50 W resin (H^+ form, x8, 20–50 mesh) and eluting with water. The eluates (300 ml) were collected and freeze-dried, and the products re-dissolved in water (5 ml). Stability (at low temperatures) to the mildly acidic conditions was assumed, because previous work[17] had shown that it was necessary to treat *N*-acetylhyalobiouronic acid with 0.5M sulphuric acid for 1 h at 100° before deacetylation was complete, and that hydrolysis of the glycosidic bonds only occurred after longer periods. Gel filtration was carried out on columns (40 × 3.1 cm) of Bio-Gel P-2 equilibrated at 4° with 10 mM sodium phosphate buffer (pH 5.0). Elution was carried out at the same temperature with the same buffer at a flow rate of 1.5 ml/min. Fractions (5 ml) were collected and scanned by the carbazole, Morgan–Elson, and Warren assays (Figs. 3*a* and *b*). In the case of oxygen degradation, fractions corresponding to the Warren-positive peak were combined, concentrated by rotary evaporation, and re-fractionated on a

column (56 × 1.1 cm) of Bio-Gel P-2, using the 10 mM phosphate buffer (pH 5.0) as eluant at a flow rate of 0.44 ml/min. Fractions (1 ml) were collected and scanned by the above-mentioned three assays (Fig. 3*c*).

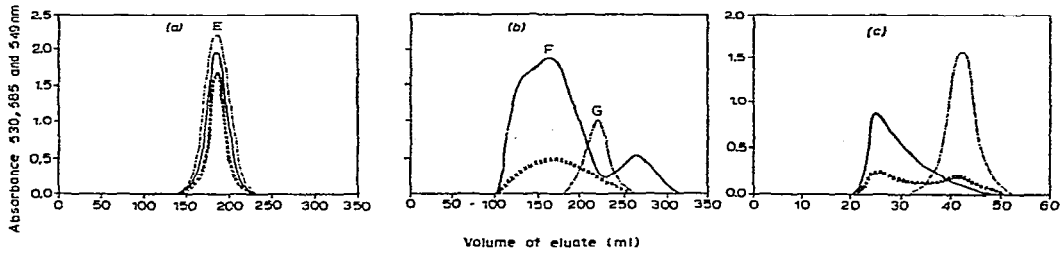

Fig. 3. Fractionation and refractionation on Bio-Gel P-2 of peak B′ (Fig. 2) from degraded hyaluronic acid; (*a*) air-degraded, (*b*) oxygen-degraded, and (*c*) further refractionation of Peak G; (———), carbazole assay; (× × × ×), Morgan–Elson assay; (–·–·–), Warren assay.

(*c*) *Analysis of the peak obtained on Bio-Gel P-2 fractionation.* Fractions corresponding to peak E obtained by Bio-Gel P-2 fractionation, in the case of air degradation, were combined, freeze-dried, and dissolved in water. Duplicate aliquots were taken and assayed by the carbazole, Morgan–Elson, and Warren methods using D-glucurono-6,3-lactone, 2-acetamido-2-deoxy-glucose, and *N*-acetylneuraminic acid as standards. The molar ratios of glucuronic acid, 2-acetamido-2-deoxy-D-glucose, and β-formylpyruvic acid were 10:10:1.

Further aliquots were hydrolysed with 2M hydrochloric acid at 100° for 12 h and then evaporated to dryness after addition of excess water. *O*-Trimethylsilyl derivatives were prepared by dissolution and equilibration in 0.2% lithium perchlorate trihydrate in redistilled pyridine (250 μl) at 37° for 2 h, followed by addition of trimethylsilyl chloride (25 μl) and hexamethyldisilazane (50 μl). After a further 10 min at 37°, g.l.c. was carried out at 210° with Silicone Ester 30 (10% on Celite) as stationary phase, nitrogen as carrier gas, and a flame-ionization detector. Standards used were 2-amino-2-deoxy-D-glucose hydrochloride, D-glucurono-6,3-lactone, sodium D-glucuronate, and 2-acetamido-2-deoxy-D-glucose which had been treated with 2M hydrochloric acid at 100° for 12 h. Peaks corresponding to 2-amino-2-deoxy-glucose and glucuronic acid arose from the fractionated material.

DISCUSSION

Since the purpose of this work was to investigate the degradation of hyaluronic acid, it was important to use hyaluronic acid that had not already been degraded. Samples *A* and *B* both showed evidence of having been degraded since they gave a positive response in the Warren assay (λ_{max} 549 nm), indicative of formation of β-formylpyruvic acid on periodate oxidation. The residues giving the response could have arisen during the alkaline extraction step used in the purification process to effect

a β-elimination from appending protein. Such alkaline treatment could have degraded the hyaluronate by further elimination reactions to give chains containing terminal, non-reducing 4-deoxy-L-*threo*-hex-4-enosyluronic acid residues. These in turn would yield β-formylpyruvic acid on periodate oxidation. Alternatively, the degradation could have been affected by metal ions present as contaminants during the extraction and purification processes. Since sample *C* showed no such prior degradation, it was selected for this work.

Viscometric studies indicated that, under the conditions presently used, considerable degradation of the hyaluronic acid occurred in the presence of air. This degradation was further illustrated by the response of the final, degraded product in the Warren assay, indicating the formation of β-formylpyruvic acid on periodate oxidation. The result was obtained in spite of the fact that the presence of ferrous ions decreased the colour yield, as has been observed elsewhere in the Warren assay of *N*-acetylneuraminic acid[20].

Furthermore, the number of alkali-titratable groups increased during the degradation. A similar effect has been noted during degradation of hyaluronic acid with ascorbic acid[21]. In contrast, no terminal, reducing 2-acetamido-2-deoxy-D-glucopyranose residues were detected by the Morgan–Elson assay, in which ferrous ions had no effect at the concentration afforded by the degradation. Other researchers[14,22] have observed that the products of degradation of hyaluronic acid with ferrous ions or ascorbic acid are not dialyzable, that there is no loss of D-glucuronic acid or 2-amino-2-deoxy-D-glucose, and that only a small number of reducing ends are released.

The production of Warren-positive material was shown to be dependent upon the presence of oxygen during the degradation, and a greater amount was produced when pure oxygen, rather than air, was used. After treatment of the hyaluronic acid with ferrous ions under three different atmospheres, and subsequent enzymic treatment, fractionation on an anion-exchange resin gave three different elution patterns (Fig. 1). The enzymic treatment applied is known to cleave intact hyaluronic acid to D-glucuronic acid, 2-acetamido-2-deoxy-D-glucose, and 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-D-glucose (the repeating unit of hyaluronic acid)[17], none of which give a response in the barbituric acid assay.

Peak A (shown by each fractionation) corresponded to non-absorbed material, had an absorption maximum of 485 nm, and was due to the enzymes, each of which was shown to be eluted in this position. Comparison of the three fractionation patterns confirmed that, when hyaluronic acid is treated with ferrous ions under nitrogen, none of the degradation products formed when oxygen was present were produced. The oxygen dependence of similar degradations has been observed by others[9,21,23,24]. Furthermore, there was a difference between the fractionation patterns for the degradations carried out under air and pure oxygen.

This difference was confirmed in degradations on a preparative scale which allowed further examination of the products. The barbituric acid assay response of peaks A′ (Fig. 2) was attributed, as before, to the presence of the enzymes used. The

Morgan–Elson assay response was attributed to the presence of free 2-acetamido-2-deoxy-D-glucose, formed by the action of β-D-acetamidodeoxyglucosidase on the parts of the hyaluronic acid chain unaffected by the ferrous treatment, and known to be eluted in this position[17]. Fractionation of the original hyaluronic acid under the same conditions gave two peaks, one of which corresponded to the positions of A′. The positive responses of peaks A′ in the carbazole test are therefore explained in terms of the presence of material of high molecular weight which is resistant to enzymic cleavage as a result of modification by the degradation process. The molar ratios for uronic acid–reducing 2-acetamido-2-deoxy-D-glucose for the air and oxygen reactions were 1:18 and 4:1, respectively, suggesting that, in the former case, little polymer was left undegraded, whereas, in the latter, a larger proportion of polymeric material remained. Any slight response in the barbituric acid assay at λ_{max} 506 nm would have been masked by the high concentration of λ_{max} 485 nm in the peak.

Peaks B′ and C′ both had greater absorptions at 505 nm than at 480 nm, indicating formation of β-formylpyruvic acid rather than malonaldehyde. As established by previous work[17], peaks B′ occurred in the elution position of 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-D-glucose and 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose. However, the second peak obtained on similar fractionation of intact hyaluronic acid also came in the position of B′. The molar ratios for uronic acid–reducing 2-acetamido-2-deoxy-D-glucose in the air and oxygen reactions were approximately 1:1 and 10:1, respectively, suggesting that peak B′, in the latter case, contained polymeric material.

Peak C′ occurred in the position of D-glucuronic acid and the normal tetrasaccharide derived from hyaluronic acid. Since 4-deoxy-α-L-*threo*-hex-4-enosyluronic acid is considered to be unstable, and in view of the similar elution position of the hyaluronic acid disaccharide and the corresponding unsaturated disaccharide[17], peak C′ probably corresponds to the hyaluronic acid tetrasaccharide in which the terminal, non-reducing uronic acid residue is unsaturated. The lack of response in the Morgan–Elson assay is attributed to modification by the degradation. A further peak D (positive response with barbituric acid) was observed in the elution position of the hyaluronic acid hexasaccharide.

In the case of degradation under air, gel-filtration chromatography of peak B′ showed that it apparently contained a single component which was eluted in the position expected for a disaccharide. Quantitative assays of this material gave molar ratios of 10:10:1 for D-glucuronic acid, terminal reducing 2-acetamido-2-deoxy-D-glucose, and β-formylpyruvic acid, respectively. That 2-amino-2-deoxyglucose and glucuronic acid were components was confirmed by hydrolysis and g.l.c. From these data, it was deduced that the material was a mixture of 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-D-glucose and 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose, the former arising from the normal enzymic cleavage of undegraded parts of the hyaluronic acid chain, and the latter from the sites of degradation of hyaluronic acid by ferrous ions. Attempts to demonstrate further the presence of an unsaturated molecule by u.v. spectroscopy

were unsuccessful on account of the very small proportion of such material produced by the degradation.

For the degradation under oxygen, gel-filtration chromatography of peak B′ showed a more complex pattern. As predicted earlier, some polymeric material was present and was obviously heterogeneous with respect to molecular size (peak F). This heterogenity explains the non-symmetrical shape of peak B′ (Fig. 2*b*). The pronounced peak G (barbituric acid assay) occurred in the disaccharide position and refractionation of this material further separated it from the polymeric material (Fig. 3*c*). The molar ratio of β-formylpyruvic acid–2-acetamido-2-deoxy-D-glucose was 0.82:1. From these data, it is assumed that this component was identical to the unsaturated disaccharide component in peak B′ obtained in the case of degradation under air.

The degradation of hyaluronic acid by ferrous ions is assumed to be of a free-radical nature. Although direct evidence is not yet available, indirect evidence (such as the requirement for oxygen shown in our present work, which is supported by other workers[9,21,23,24], and the inhibition of degradation by compounds that are known to inhibit free-radical reactions[15]) supports the postulation of the radical mechanism. Thus the actual reaction taking place is probably free-radical degradation by ferrous ions, of which a β-elimination mechanism is a part. The β-elimination probably involves loss of H-5 from the glucuronic acid moiety, because the resulting radical would be stabilized by resonance interaction with the carboxyl group[25]; Pigman[22] has suggested that C-4 is the most susceptible site in the glucuronic acid moiety.

The present recognition of modified uronic acid residues indicates that 2-acetamido-2-deoxy-β-D-glucosyl bonds were cleaved in the degradative process. Therefore, the absence of concomitant production of terminal reducing 2-acetamido-2-deoxy-D-glucose residues suggests that the 2-amino-2-deoxy-D-glucose residues involved also became modified in the degradative process. Worthington[26] has proposed a free-radical mechanism for the photodegradation of cellulose in which a lactone is one of the products. A similar process could have taken place in the present system, resulting in the negative Morgan–Elson assay after ferrous degradation.

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