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Gel Chromatographic Behavior of Aqueous Ferric Nitrate*

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Summary

Ferric nitrate solutions with different degrees of hydrolysis were investigated on polydextran gels having various exclusion limits. Typically chromatograms exhibited two well-resolved peaks, one at the void volume and the other at the total liquid volume of the gel. The respective peaks corresponded to higher and lower molecular weight fractions of the iron in the injected sample. Similar behavior was observed in "negative-peak" investigations where the positions of sample and eluent were reversed. Frontal analysis of the iron solution showed two breakthroughs corresponding to excluded iron moving in the void volume and admitted iron moving near the total liquid volume of the gel. The molecular size distribution of the ferric nitrate samples was studied as a function of OH–Fe ratio by noting changes in the percentage of iron in the respective fractions for gels having known exclusion limits.

INTRODUCTION

For the past decade biochemists and polymer chemists have been studying the separation of organic macromolecules under the names "gel filtration" and "gel permeation chromatography," respectively. It has been suggested (*1*) that the term "gel chro-

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matography" be used to include all techniques for separating molecules on the basis of their dimensions by passing a solution through a bed of gel grains. Pecsok and Saunders (*2*) have consolidated what is presently known about gel chromatography in an excellent review article.

Recently a paper describing the separation of stable polyphosphates on a polydextran gel appeared in the literature (*3*). It demonstrated that gel chromatography can indeed be useful for investigating inorganic, as well as organic, polymeric systems. A detailed investigation of inorganic systems is currently underway in our laboratories (*4*), and results indicate that a general theory for gel chromatography must account for electrostatic effects in addition to size effects. It will also be evident that present theory is not adequate to explain the data reported in this paper for the ferric nitrate system.

The basic equation describing the operation of a gel column has been given by Gelotte (*9*) as

$$V_e = V_o + K_d V_i$$

where V_e is the peak volume of the eluted solute particles, V_o the volume of solvent outside of the gel phase (void volume), and V_i the volume of unbound solvent inside of the gel phase. K_d is a distribution coefficient which characterizes the sample in the following manner:

$K_d = 0$: particles are excluded from the gel phase
$K_d = 1$: particles are small enough to diffuse freely within the gel
$0 < K_d < 1$: particles penetrate the gel phase but cannot diffuse without some restriction
$K_d > 1$: particles interact strongly with the gel (adsorption and ion exchange)

Appearance of a peak in the region between the void volume and total liquid volume ($0 < K_d < 1$) does not necessarily signify the absence of specific interactions.

Several authors (*5–8*) have published on the hydrolytic behavior of ferric iron. It is generally agreed that polymerization begins with the formation of $Fe_2(OH)_2^{4+}$ and continues with time to the precipitation of ferric hydroxide. Spiro et al. (*8*) isolated the fraction of a hydrolyzed ferric nitrate sample which was excluded from a

Sephadex G-25 gel column and found evidence for a high-molecular-weight polycation.

EXPERIMENTAL DETAILS

Reagents and Solutions

An 0.2 *M* stock solution was prepared by dissolving a weighed quantity of reagent ferric nitrate (Mallinckrodt Chemical Co.) in distilled water. Samples with varying degrees of hydrolysis were prepared by adding weighed amounts of reagent potassium bicarbonate (J. T. Baker Chemical Co.) to the stock solution and then bubbling for a few minutes with nitrogen to remove the dissolved carbon dioxide. This method of adding base prevented the local exceeding of the solubility product of ferric hydroxide. Up to 2.5 equivalents of base can be added in this manner before ferric hydroxide precipitates.

The hydrolysis was accompanied by a reddening of the solution and appeared to be largely complete within 15–30 min after adding the bicarbonate. These sample solutions were clear and stable (contained no visible precipitate) for 2–5 days, depending on the degree of hydrolysis. It is interesting to note that the solutions which contained the highest OH–Fe ratio were the most stable with respect to precipitation.

Sephadex gels (Pharmacia, Inc., Piscataway, N.J.) were used almost exclusively in this investigation because they swelled in aqueous solution and were available in a wide variety of pore sizes. Sephadex G-10, G-15, G-25, G-50, G-75, and G-100 gels were packed in 1 × 18 cm glass columns equipped with T joints and septum inlets for on-stream injection.

Procedures

Typically samples were injected via a 50-μl syringe (Hamilton Company, Whittier, Cal.). Two variable-speed, positive-displacement pumps (Sigmamotor, Inc., Middleport N.Y., and Durrum Instrument Corp., Palo Alto, Cal.) were used interchangeably to force eluent through the column and detector. Two detectors were available for on-stream monitoring of the eluent stream: (1) a differential refractometer (Waters Associates, Framingham, Mass.) and (2) a modified Beckman DU spectrophotometer with flow cell. The spectrophotometer was set at 250 mμ to detect iron in the eluent stream.

The void volume V_0 was determined for all freshly prepared columns by eluting a sample of Blue Dextran (Pharmacia, Inc., Piscataway, N.J.). The total liquid volume, $V_0 + V_i$, was determined for all but the G-10 columns by eluting a sample of nitric acid. The total liquid volume of the G-10 columns was determined by eluting a sodium chloride sample because there was some evidence (*4*) that nitric acid was partially excluded from the pores in this highly crosslinked gel.

In addition to the usual chromatographic and frontal analysis procedures, a "negative-peak" method was utilized. In that procedure an iron solution was used as eluent and samples were injected onto the ferric nitrate background. Under those conditions the sample and/or eluent could be characterized not only by the elution volume of the peaks, but also by the positive or negative deflection from the base line.

In general it was found that both the molar absorptivity and molar refractive index varied with the degree of hydrolysis of a sample. The change in the molar absorptivity was due to differences in the ultraviolet absorption spectra of the various iron species in the sample, and the change in the molar refractive index was primarily due to the addition of potassium bicarbonate which could not be distinguished from iron by the refractometer. As a result the correlation of peak heights or areas with the percentage of total iron was not reliable. Instead a colorimetric method (*10*) was used to accurately determine the amount of iron under a given peak. The eluent containing the iron was collected in a volumetric flask and aliquots of hydrochloric acid and potassium thiocyanate were added. Because of the slow depolymerization of the hydrolyzed iron, the collected peaks were allowed to remain standing in the hydrochloric acid for a few hours before adding the thiocyanate. Sample sizes were selected so that the absorbance reading on a Bausch and Lomb Spectronic 20 was equal to the fraction of the total iron under the peak collected for analysis.

RESULTS

Figure 1 shows typical chromatograms which were obtained for the injection of iron samples with different degrees of hydrolysis

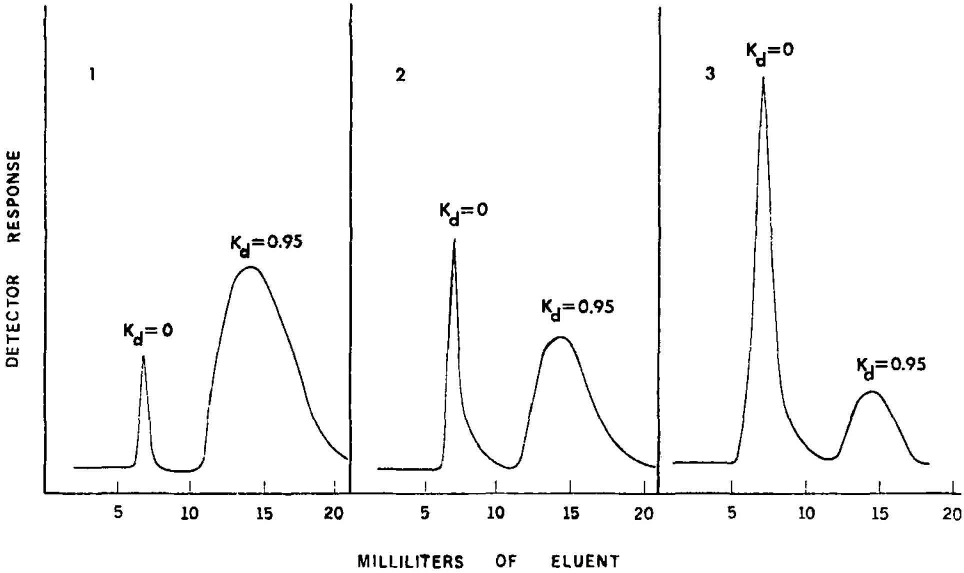

FIG. 1. Chromatograms obtained for 25 μl of 0.2 *M* ferric nitrate with (1) OH–Fe = 1.5, (2) OH–Fe = 2.0, and (3) OH–Fe = 2.5 on a G-25 gel column eluted with nitric acid.

onto a Sephadex G-25 gel column. The eluent was 0.01 *M* nitric acid and the spectrophotometer was used to monitor the eluent stream. It is evident from Fig. 1 that the samples were separated into two fractions by the action of the gel column. The narrow fraction was dark red in color and eluted exactly in the void volume of the column ($V_e = V_o$, $K_d = 0$). The broader fraction was light yellow and appeared in slightly less than the total liquid volume of the column ($K_d = 95$).

Under the nitric acid eluent conditions, 100% recovery of unhydrolyzed ferric nitrate (OH–Fe = 0) and 90–100% recovery of the more extensively hydrolyzed samples was found.

In the absence of specific interactions such as adsorption and ion exchange [the ion-exchange capacity of Sephadex G-10 has been shown in these laboratories (*4*) to be 30–60 μeq/g], such data would indicate that two discrete molecular-weight fractions were present in the original sample. High-molecular-weight iron, which was excluded from the gel, would be assigned to the peak at the void volume and low-molecular-weight iron, which was free to

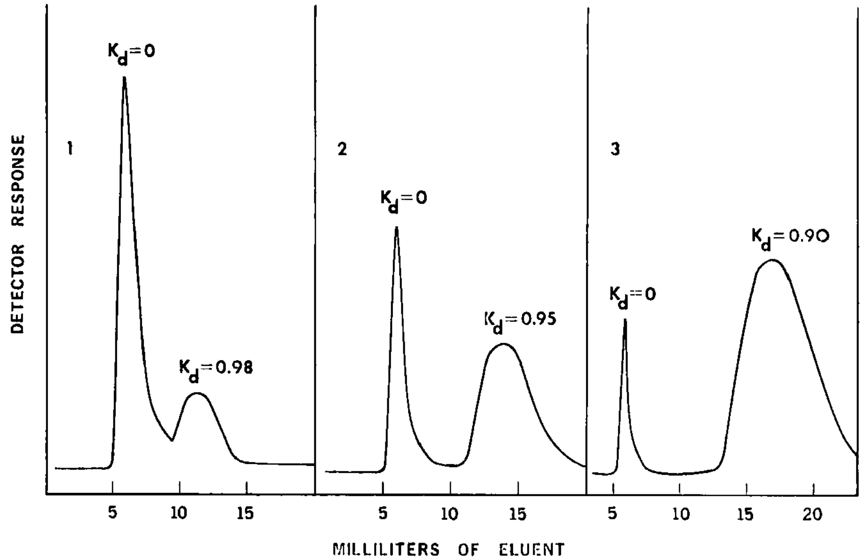

FIG. 2. Chromatograms obtained for 25 μl of 0.2 *M* ferric nitrate with OH–Fe = 2.0 on (1) G-10, (2) G-25, and (3) G-50 gel columns eluted with nitric acid.

move within the gel matrix, would be assigned to the peak at the total liquid volume.

Spiro et al. (8) have shown the presence of a polydisperse polymer fraction in hydrolyzed ferric nitrate solutions by analytical ultracentrifuge studies. They demonstrated, using Schlieren patterns of ultracentrifuged samples, that the amount of polymer increased with increasing degree of hydrolysis. This is also evident from the chromatograms in Fig. 1.

In addition to the study on G-25, iron samples with OH–Fe = 0, 1.0, and 2.0 were investigated on Sephadex G-10, G-15, G-50, G-75, and G-100 gels. Figure 2 exhibits typical chromatograms obtained for a sample of ferric nitrate having OH–Fe = 2.0 on gels with various pore sizes. It can be seen from the change in relative peak areas that part of the iron which was excluded from the smaller pore gels appeared near the total liquid volume of the next larger pore gel. This behavior eliminated the possibility that the gel chromatograms reflected a true molecular size distribution and suggested perhaps that an adsorption or depolymerization process had occurred which operated primarily in the pores of the gel particles.

It is interesting to note, however, that the peak resolution increased with larger pore-size gels. This is consistent with gel theory which predicts that the resolving power of a column of a given length should be directly related to the pore volume.

The fractions which contained the respective peaks were collected and their UV-visible spectra were taken at various time intervals after they emerged from the column, as shown in Fig. 3. Mulay and Selwood (*11*) have studied the UV-visible adsorption spectra of hydrolyzed and unhydrolyzed ferric iron in perchloric acid solution. They reported adsorption peaks in the vicinity of 240 and 335 mμ for the low-molecular-weight iron species predominant at pH 1.0 (monomer and dimer). Above pH 1.8 they found a broad absorption, with no definite peaks, which extended into the visible region of the spectrum. Turner and Miles (*12*) also reported absorption peaks at 240 and 300 mμ for pH values where low-molecular-weight iron would be predominant. The spectra in Fig. 3(A) show that the iron fraction which eluted in the void volume absorbed broadly into the visible region and, therefore, probably contained predominantly high-molecular-weight iron species. The

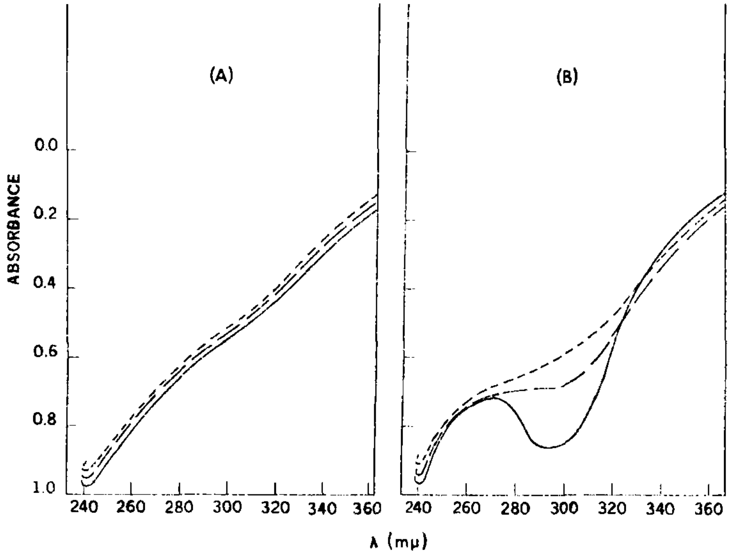

FIG. 3. Spectra of the fractions collected at the void volume (A) and at the total liquid volume (B) at various time intervals after emerging from the column. Solid lines, 0 hr; dashed lines, 24 hr; dotted lines, 48 hr.

spectra in Fig. 3(B) show that the fraction eluted in the total liquid volume exhibited an absorption peak at 300 mμ and therefore contained predominantly low-molecular-weight iron species. The spectra also show that the excluded fraction was stable for at least 48 hr after it had been isolated, while the low-molecular-weight fraction hydrolyzed rapidly. This effect could also be observed visually. The sample was fractionated on the column into a fast-moving red band and a slower-moving light yellow band. After emerging from the column, the dark red fraction did not change color on standing, while the clorless fraction quickly became more yellow as it hydrolyzed. Spiro et al. (8) also reported that, while the hydrolysis of ferric nitrate proceeded rapidly, the polymer fractions which were excluded from a G-25 gel were very stable with respect to precipitation of ferric hydroxide. We also found that isolated polymer fractions were stable for several weeks, as compared to 2–5 days for unfractionated samples.

The percentage of iron corresponding to the respective peaks was determined for a variety of different pore-size gels. The results are given in Table 1. A plot of the percentage of excluded iron

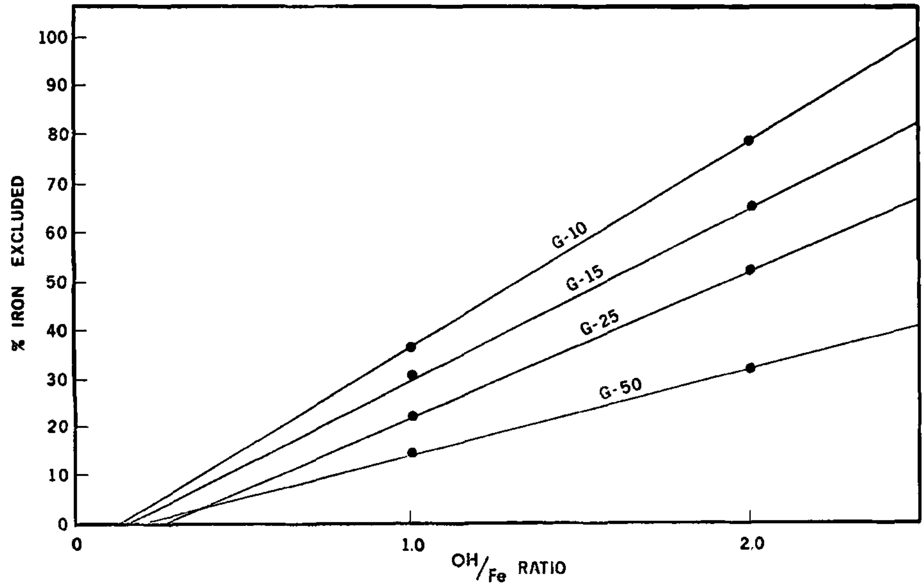

FIG. 4. The percentage of iron excluded from various gels versus degree of hydrolysis.

TABLE 1

Percentage of Iron in the Respective Peaks for Various Gels and Different Degrees of Sample Hydrolysis

Sephadex gel[a]	OH–Fe ratio	% Fe excluded, peak 1	% Fe admitted, peak 2[b]
G-10	0	0	100
	1.0	36	64
	2.0	75	25
G-15	0	0	100
	1.0	30	70
	2.0	64	36
G-25	0	0	100
	1.0	21	79
	2.0	41	59
G-50	0	0	100
	1.0	13	87
	2.0	21	79
G-75	0	0	100
	1.0	1	99
	2.0	6	94
G-100	0	0	100
	1.0	0	100
	2.0	1	99

[a] 0.01 *M* HNO_3 eluent.

[b] Includes 0–5% irreversibly adsorbed.

versus OH–Fe ratio for each gel is given in Fig. 4. It suggests that a rather wide distribution of molecular weights is present in hydrolyzed ferric nitrate solutions.

Also, the kinetics of polymer formation were investigated in a brief experiment. The growth of the excluded fraction was followed as a function of time, as shown in Fig. 5. It shows that the hydrolysis is rapid, being nearly complete in 15–30 min, and demonstrates a possible general application of the gel chromatography.

Frontal analysis of ferric nitrate samples showed two breakthroughs corresponding to excluded iron moving in the void volume and admitted iron moving in the total liquid volume of the gel. The iron solution to be studied was introduced as a front onto a gel column which had been equilibrated with dilute nitric acid, and the eluent stream was monitored with the differential refractometer. The "reverse" of the iron breakthrough was then observed

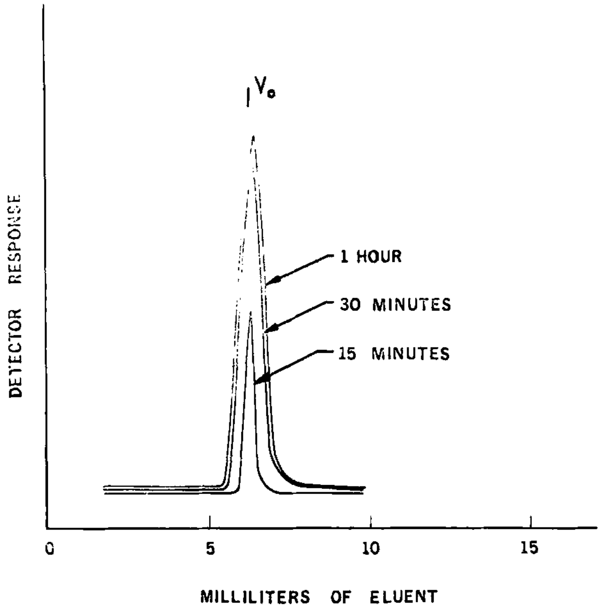

FIG. 5. Kinetics of polymer formation by monitoring the excluded fraction of a G-25 gel column as a function of time after addition of potassium bicarbonate (OH–Fe = 2.0).

by flushing the gel with dilute nitric acid while still monitoring the eluent. A typical set of frontal analysis curves is shown in Fig. 6 for an 0.01 *M* ferric nitrate solution with OH–Fe = 1.0. The breakthrough volumes were identical, within experimental error, to the appearance volumes of the respective peaks in the corresponding gel chromatograms. The figure also shows that, with the dilute nitric acid eluent, the relative amounts of excluded and admitted iron were the same at the front and back of the iron sample.

Iron solutions were also investigated by a negative-peak method in which the position of sample and eluent were reversed. A G-15 Sephadex gel column was eluted with an 0.01 *M* iron solution which contained both excluded and admitted species; then samples of water and ferric nitrate were introduced. Again, two peaks were observed at the void volume and total liquid volume, respectively. A chromatogram exhibited two negative peaks when the sample was less concentrated in both excluded iron and admitted iron than the eluent, one negative and one positive peak when either excluded or admitted iron concentration in the sample was less than that in the eluent, and two positive peaks when the sample contained more of both types of iron than the eluent. The typical chromatograms shown in Fig. 7 demonstrated that there were only two iron fractions traveling down a column. As was also the case in normal elution chromatography using nitric acid alone as the eluent, the peak at the void volume was sharper than the peak at the total liquid volume, but both peaks were very symmetrical.

When distilled water was substituted for dilute nitric acid as the eluent in a normal gel chromatographic experiment, two well-defined peaks were again observed at the void volume and total liquid volume, respectively. It was discovered, however, that a large amount of iron was being irreversibly adsorbed onto the column under those conditions. The gel columns rapidly stained to

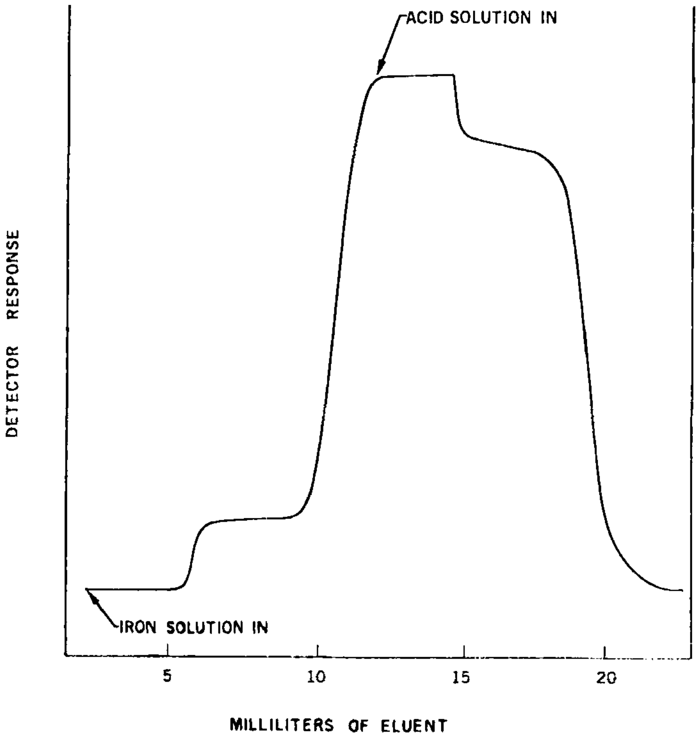

FIG. 6. Frontal analysis of 0.01 *M* ferric nitrate with OH–Fe = 1.0 on a G-15 gel column eluted with dilute nitric acid.

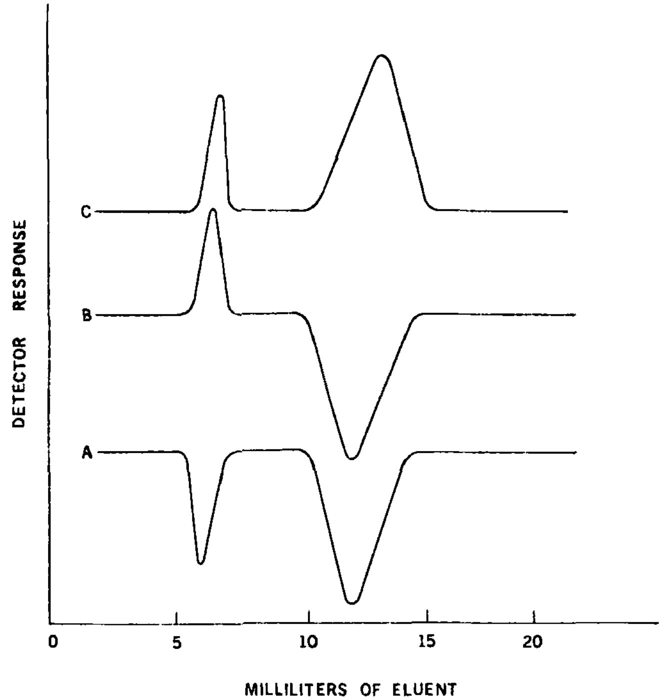

FIG. 7. Chromatograms observed on a dilute ferric nitrate background when the sample contained less of the excluded and admitted iron (A), more of the excluded but less of the admitted iron (B), and more of both excluded and admitted iron (C) than the eluent.

the orange color of hydrolyzed iron and actually became "crystalline" (i.e., hardened, granular) after repeated injections of iron samples. To obtain reproducible chromatograms with a water eluent, it was necessary to condition fresh columns with several injections of iron sample. No amount of conditioning, however, gave more than 50–70% recovery of the total iron. In general the more highly crosslinked gels such as G-10 and G-15 retained more of the sample.

The percentage of sample excluded from a given gel was always greater for a water eluent than for an acid eluent. For example, a ferric nitrate solution with OH–Fe = 0 was approximately 20% excluded from G-10, but no excluded fraction could be detected when the same column and sample were investigated with a dilute nitric acid eluent. Although there was undoubtedly some depolymerization of iron as it passed down a column, there was no chromatographic evidence that gradual depolymerization of the excluded fractions was taking place in the acid eluent. This would indicate that electrostatic effects, which depend on the ionic strength and character of the eluent, may be very important in determining effective pore diameters of gels toward charged inorganic polymers. The largest pore-size gel on which any excluded iron could be detected was G-75 for an acid eluent and G-100 for a water eluent. Andrews (*13*) has reported that G-75 and G-100 have approximate pore diameters of 70 Å and 100 Å, respectively. This agrees with the electron microscopy data of Spiro et al., who established an average molecular diameter in an extensively hydrolyzed iron solution to be 70–90 Å.

Frontal analysis studies were also carried out using an 0.01 *M*

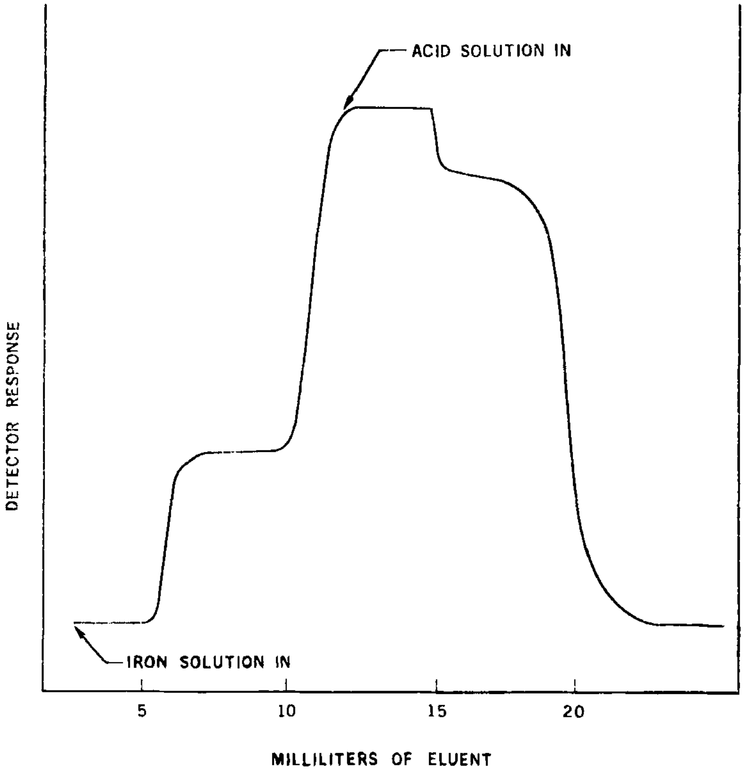

FIG. 8. Frontal analysis of 0.01 *M* ferric nitrate with OH–Fe = 1.0 on a G-15 gel column eluted with distilled water.

ferric nitrate solution with OH–Fe = 1.0 and a water eluent. The curves are shown in Fig. 8. Note that the curves are asymmetrical. The amount of excluded iron which appeared at the void volume as the iron sample was flushed in was greater than that which appeared at the void volume as the iron sample was flushed out. As the iron front moved onto the fresh gel column, the amount of excluded iron depended on the polymer size and effective pore diameter of the gel in water; but, once the iron sample had equilibrated with the column, the iron polymer and gel were then in the dilute nitric acid background of the hydrolyzed sample. The overall frontal behavior was consistent with the observation that, using conventional elution chromatography, more excluded iron was detected on a water-eluted column (where further hydrolysis and polymerization occurred) than on one eluted with dilute nitric acid.

DISCUSSION

Much evidence for the presence of high-molecular-weight iron polymers in hydrolyzed ferric nitrate solutions has been published in recent years, but rarely has such evidence been as tangible as that presented by the gel chromatograms. Iron samples were separated into two discrete fractions by the action of the gel columns. A narrow fraction eluted in the void volume of the gel, and there was spectral evidence that this fraction consisted of iron which was too large to enter the gel pores. A broader fraction eluted in approximately the total liquid volume and apparently consisted of molecules which were not only small enough to enter the gel pores but were able to move quite freely within the matrix. On further study with various gels, it became evident that the two fractions could not represent a true molecular-weight distribution in the iron system. Indeed, much of the iron that was excluded from G-10 appeared near the total liquid volume of G-15.

While normal gel theory for organic systems cannot explain the data observed for ferric nitrate, there is considerable evidence that gel filtration is important in determining the nature of the chromatograms. In Fig. 2 the peak resolution (distance between peaks in milliliters) increased with increasing pore volumes of the gels. This suggests that the iron samples were fractionated by gel chromatography rather than by ion exchange or adsorption chromatography.

The fractionation of iron samples by Sephadex gels can be viewed as gel filtration, but it is clear that some of the rules must be modified to account for the two discrete fractions. Possibly iron species small enough to get into the gel matrix are exposed to strong interactions that depolymerize them rapidly to species small enough to be eluted in the total liquid volume of the column. It is also possible that adsorption and interactions with the fixed negative charges in the gel matrix combine to retain the smaller species.

The key to interpeting gel chromatographic data on ionic systems may be to use a three-dimensional charged membrane as a model for the gel column. The true picture for the ferric nitrate system is probably a combination of gel filtration, Donnan effects, and depolymerization.

We have shown that gel chromatography can be useful for studying relative molecular size distributions in the ferric nitrate system. In addition, because the iron species range in color from light yellow to dark red, gel chromatography of iron(III) samples is uniquely applicable to simple visual demonstration experiments.

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