

A Study on Fractionation and Characterization of Water-Soluble Natural Fe-Chelates From Garbage Compost and Activated Sewage Sludge.

Nae-Joung Park W,L, Lindsay*

College of Science and Engineering, Hong-Ik University

(Received May 11, 1975)

活性汚泥 및 塵芥堆肥中 水溶性 鐵 킬레이트의 分離와 特性에 關한 研究

朴來正·윌라드 엘 린지*

弘益大學校 理工大學

(1975년 5월 11일 수리)

摘 要

이 研究는 都市有機廢物을 利用한 有機質肥料로서 活性汚泥 및 塵芥堆肥를 킬레이트화 물질로서 利用하기 爲해서 이들 物質의 特性을 調査하였다.

鐵킬레이트화에 關與하는 水溶性物質을 분자체 分劃法으로 分離하여 紫外線 및 赤外線分光 스펙트럼에 의해서 그 構造를 調査하였고 이들 킬레이트의 安定度常數를 이온交換平衡法으로 測定하여 이들을 킬레이트 물질로 使用하였을때 그 安定性を 檢討하였다.

塵芥堆肥에서 抽出된 有機物質은 Sephadex G-25에 依해 4個分劃으로 分離되었고 이중 鐵과 킬레이트를 이루고있던 分劃은 分子量이 5000~10,000 사이에 있었고 Polyphenol構造의 酸素群이 킬레이트화에 關與하고 있었다.

活性汚泥中 可溶性 物質은 Sephadex G-25에 依해 6個의 分劃으로 分離되었고 이중 킬레이트를 이루고 있던 分劃은 5000~10,000 또는 5000에 약간 未達하는 分子量을 가지고 있고 Polypeptide의 amide기가 킬레이트화에 關與하였다. 이들의 安定度常數도 合成킬레이트 물질의 安定度常數와 큰 差異가 없을 程度로 安定하였다.

INTRODUCTION

Utilization of organic wastes such as town refuse and sewage sludge from metropolitan area has received increased attention to solve the pollution problems of our urban environment. Numerous workers have investigated the usefulness of their materials in the aspect of fertilizers. Most of the researchers (1, 2, 3) have emphasized the value of the organic wastes as major nutrient sources as soil amendments.

However, some workers (1, 4, 5) advocated those materials as micronutrient sources.

Recently, Park and Lindsay (6) proved the effectiveness of garbage compost and activated sewage sludge as chelating agent sources. O'Connor et al. (7) also found that diffusion of Fe in the soil increased linearly with concentration of water-soluble chelated Fe suggesting the importance of water solubility of chelates with respect to Fe availability in soil.

This experiment aimed at investigating the properties of the water-soluble natural chelating

*Professor, Colorado State University, U.S.A.

agents from garbage compost and activated sewage sludge responsible for Fe chelation.

The water-soluble fractions of these materials were fractionated by means of Sephadex gel filtration and the fractions of Fe chelates were traced by radioactive ^{59}Fe .

The fractions were examined by UV and IR spectroscopy, and stability constants for Fe was also studied exchange equilibrium method.

MATERIALS AND METHODS

Two solid organic waste materials were used in this study. One was a garbage compost obtained from the Richland Composting plant near Boulder, Colorado. This material was processed by an aerobic composter, capable of transforming 100 tons of incoming garbage wastes per day into odorless product.

The other was a dried activated sewage sludge processed by adding FeCl_3 and lime to facilitate flocculation of the suspended organic materials prior to vacuum filtration at the Metropolita Denver Sewage Disposal District in Denver, Colorado.

Preparation of Samples

Water extracts were prepared by placing 400g of air-dried solid wastes into a 5-liter Erlenmeyer flask and adding four liters of de-ionized water.

The suspensions were then filtered through Whatman No. 2 filter paper, and the filtrate were stored in a refrigerator during the experiment.

Sephadex Column Fractionation

Medium grade Sephadex G-25 and G-50 were suspended in distilled water and packed in a Sephadex column (1.9cm \times 90cm). The void volume was determined with 0.2% Blue Dextran 2,000 (Pharmacia Fine Chemicals, Inc.).

A 20 ml-aliquot of sewage extract or a 40 ml-aliquot of compost extract was concentrated to 2ml by means of a rotatory evaporator under vacuum at $50 \pm 5^\circ\text{C}$. To trace the active chelating agents, 1 μc of ^{59}Fe was added before concentration. The concentrated extracts were added to the column and eluted with 0.02M $(\text{NH}_4)_2\text{CO}_3$ buffered at pH 7.9. This pH corresponded to the pH of the soil used in the greenhouse study.

Two to three milliliters eluates were collected in the test tubes using a fraction collector.

Absorbance of ultraviolet light at 260 m μ was determined in each collection tube using a Beckman DB spectrophotometer. Also 2ml of eluates were taken from each collection tube, and the radioactivity of ^{59}Fe was counted in a well-type scintillation counter for one minute.

UV and IR absorption Spectroscopy

The ultraviolet absorption spectrum of each fraction separated by Sephadex G-25 was determined over the range of 300 to 220 m μ using a Beckman DB spectrophotometer. The effect of pH on the absorption maxima of a few fractions from sewage extract was examined in 1 N HCl and 1N NaOH solutions.

Measurement of Stability Constants

The principle of resin-exchange equilibrium was used to determine the stability constants of metal complexes. This method is based on the fact that a quantity of metal bound to a given weight of resin at equilibrium, MR, is proportional to the concentration of free ions, (M), in solution. Martell and Calvin (8) reported the following relationship for determining stability constant of water-soluble chelates:

$$\log(\lambda_0/\lambda - 1) = \log K + x \log(\text{ch})$$

where $\lambda_0 = \frac{\text{MR}}{(\text{M})}$ is the distribution constant in absence of chelating agent.

$\lambda = \frac{\text{MR}}{(\text{M}) + (\text{MCH})}$ is the distribution constant in presence of chelating agent.

$K = \frac{(\text{MCH})}{(\text{M})(\text{CH})^x}$ is the stability constant of the complex.

x is the number of molecules of complexing agent which combine with 1 mole of metal, and,

(CH) is the concentration of the complexing agent in moles per liter.

The extracts of garbage compost and sewage sludge were purified by passing them through Amberlite IR-120 cation-exchange resin. The stock solution contained 6.5 and 5.3mg organic matter per ml of compost and sewage extracts, respectively. A 10-ml aliquot of stock solution was used to determine the influence of pH on the chelating

ability of organic compounds. A 2.5ml of 1N KCl and 1ml of FeCl_3 solution containing 100 ug Fe and 1 uc ^{59}Fe were added. The pH was adjusted to 4.0 with either 0.1 N KOH or 0.1 N HCl. The mixture was diluted to 25ml and transferred to a 50-ml Erlenmeyer flask, and 0.5g K-saturated resin Amberlite IR-120 (15-20 mesh) was added. The suspension was equilibrated by overnight shaking at $25 \pm 2^\circ\text{C}$.

Ferric iron precipitated with pH adjustment and thus interfered with the measurement of stability constants of Fe^{3+} . But the stability constants for Zn chelates from which the strength of chelation with Fe could be presumed were successful using the same procedure attempted for Fe. Aliquots of 2, 4, 6, 8, and 10 ml of compost extract and 3, 6, 9, 12, and 15 ml, of sewage extract with blank were taken.

A 2.5 portion of N KCl and 10 ug of Zn containing 1 uc ^{65}Zn were added to each aliquot and the pH was adjusted to 4.0. The mixture was made up to 25ml, and 0.5g of K-saturated resin was added.

One hour shaking was sufficient to attain equilibrium.

The concentration of chelating sites was measured by adding excess metals to precipitate the metal-complexes as suggested by Randhawa and Broadbent (9). Ten milligrams of Zn as ZnSO_4 , and 2.5 ml of 1N KCl were added to 5 ml aliquots of extracts. The pH of the resulting solution was adjusted to 4.0, and the volume was made up to 25 ml. The suspension was centrifuged, and the concentration of Zn remaining in the solution was measured.

RESULTS AND DISCUSSIONS

Sephadex Column Fractionation

Sephadex G-25 separated the compost extract into four major fractions according to UV absorption at 260mu (Fig. 1) Fraction I appeared at the void volume of Sephadex G-25, which has a molecular weight fractionation range of about 100 to 5,000. Since the molecular configuration of compounds in the water extracts is not known, it was not possible to determine exact molecular

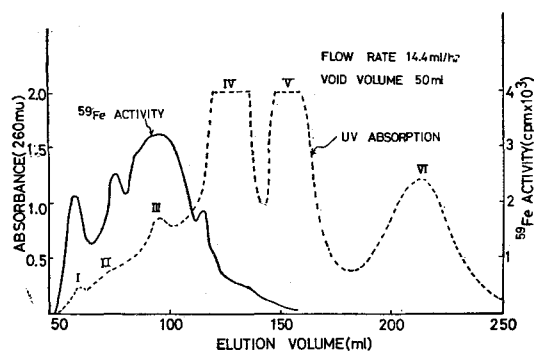


Fig. 1. Fractionation of water-soluble organic materials from garbage compost with its associated ^{59}Fe activity using Sephadex G-25.

weights for the fractions by calibrating with known standards. However, according to the general exclusion limit of Sephadex G-25, the molecular weight of fraction I probably exceeds 5,000.

Almost all the added ^{59}Fe was associated with fraction I. When Sephadex G-50 was used for further separation of fractions from the void volume (Fig. 2), most of the organic matter as well as ^{59}Fe appeared at elution volume 160 to 180ml. The results indicate that chelating agents

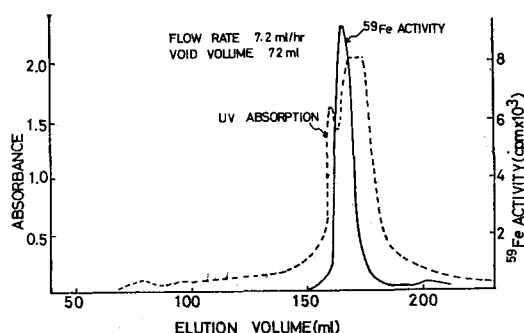


Fig. 2. Fractionation of water-soluble organic materials from garbage compost with its associated ^{59}Fe activity using Sephadex G-50.

from garbage compost responsible for Fe chelation have molecular weights ranging from 5,000 to 10,000.

Sewage extract showed six major fractions when separated with Sephadex G-25 (Fig. 3). A majority of the Fe chelates showed molecular weights below 5,000. Only a small fraction app-

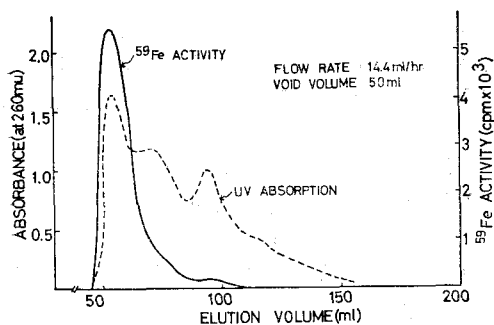


Fig. 3. Fractionation of water-soluble organic materials from sewage sludge with its associated ^{59}Fe using Sephadex G-25.

appeared at the void volume (fraction I). By fractionation with Sephadex G-50 (Fig. 4), most of the organic matter in sewage extract as well as ^{59}Fe appeared between elution volumes 130 and 210 ml. Again the chelating agents in fraction I

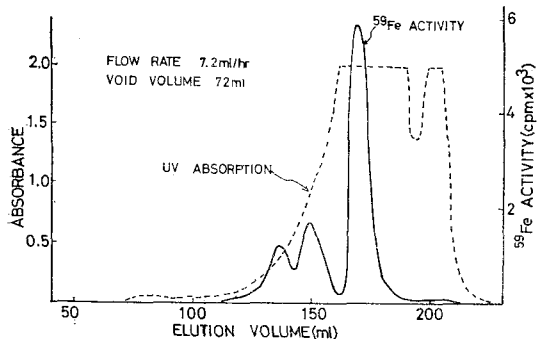


Fig. 4. fractionation of water-soluble organic materials from sewage sludge with its associated ^{59}Fe activity using Sephadex G-50.

by Sephadex G-25 represent molecular weight between 5,000 and 10,000. Discrepancy between UV absorption and ^{59}Fe radioactivity indicates that these fractions separated by Sephadex gel filtration contained heterogenous compounds with respect to Fe chelation. Karpukhin and Fokin⁽¹⁰⁾ used paper chromatography to obtain further separation of fractions separated by Sephadex.

The major chelating agents from these solid waste extracts responsible for Fe chelation are not apparently low molecular weight organic acids or amino acids as reported for soil podzolization processes^(11,12,13).

Ultraviolet Absorption Spectra

Ultraviolet absorption spectra were obtained for the water-soluble organic matter fractions of garbage compost and sewage sludge separated by Sephadex G-25.

Fractions of compost extract showed no special absorption in the near UV region (Fig. 5). Those spectra are quite similar to those reported for soil humic acids⁽¹⁴⁾.

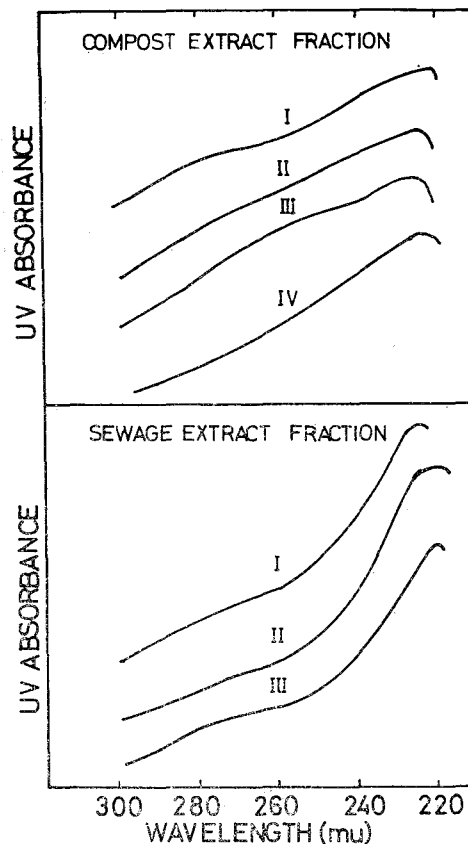


Fig. 5. UV absorption spectra of all fractions of compost extract and fraction I, II, and III of sewage extract separated by Sephadex G-25.

Humic and fulvic acids, however, begin absorbing at 700 $m\mu$ whereas the compost extract begins at 500 $m\mu$ and sewage extract at 400 $m\mu$.

Fractions IV, V, and VI of sewage extracts showed significant absorption maxima in the near ultra-violet region (Fig. 6). Fractions IV and V absorbed at 260 and 250 $m\mu$, respectively, which suggests possible association with nucleic acid

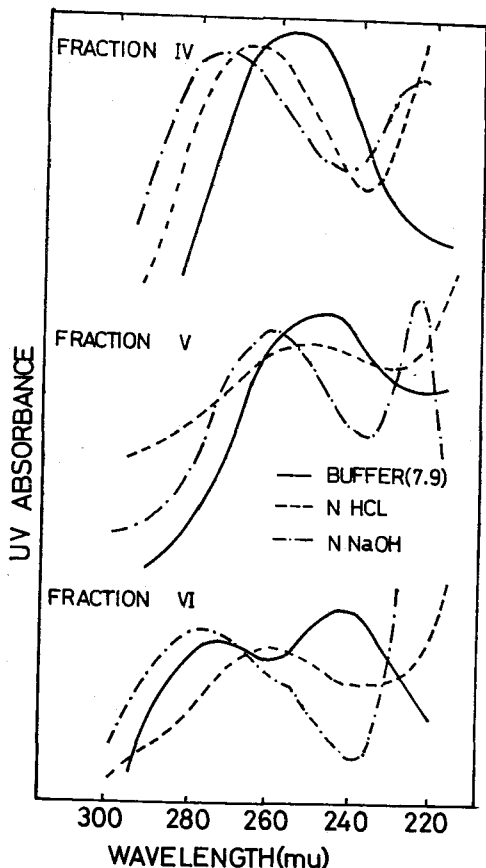


Fig. 6. UV absorption of fraction IV, V, and VI of sewage extract and pH effect.

components.

The effect of pH on the absorption maxima was also examined. Under strong acidic conditions, fraction IV and V showed bathochromic shifts, and under alkaline conditions, even greater shifts were noted. These phenomena are usually associated with nucleic acid components, mainly due to ionization of bases^(15,16).

Accordingly fractions IV and V may contain decomposition products of nucleic acid as one of the main constituents.

Fraction VI showed absorption at about 270 and 240m μ . The absorption at 270m μ seemed to indicate the presence of aromatic amino acid components⁽¹⁷⁾.

The effect of pH was examined to test for ionizable phenolic group as in tyrosine⁽¹⁸⁾. A bathochromic shift was observed in N NaOH and

hypsochromic shift in N HCl. The results suggest that fraction VI might contain the tyrosine component. The absorption at 240m μ was not identifiable and nearly disappeared by changing pH, possibly due to hypochromic effect.

Infrared Absorption Spectra

Infrared spectroscopy is one of the most rapid and convenient tools available for compound identification and structure analysis in organic chemistry and biochemistry.

Considerable effort has been spent in studying the infrared spectra of complex substances such as proteins, nucleic acids, carbohydrates, coal, and humic acids^(19,20,21,22).

The infrared spectra (4,000–650cm⁻¹) of water extracts of garbage compost and sewage sludge in KBr pellets are given in Fig. 7.

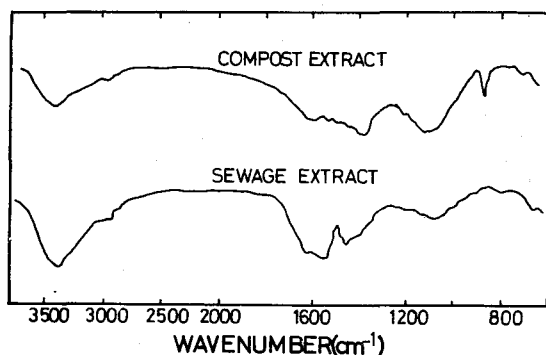


Fig. 7. Infrared spectra of water extracts of solid organic wastes.

Because of the superposition of absorption spectra of various groups in such complex molecules, the spectra were rather indefinite and broad. It was difficult to assign the absorption band to exact groups of molecular structure as already pointed out by the other workers^(23,24).

Better resolution of spectra was obtained by fractionation by Sephadex G-25. The major absorption bands of the original water extracts as well as the fractions separated by Sephadex gel filtration (Fig. 8, 9) were at 3400, 1600, and 1100cm⁻¹. These absorption maxima were similar, with a few exceptions, to those of fulvic and humic acids from soil^(25,22,26).

Kononova⁽²²⁾ concluded that the similarity of spectra, from comparative studies on humic acids.

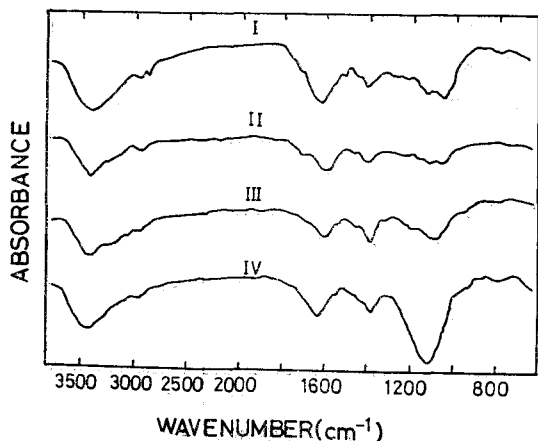


Fig. 8. Infrared spectra of fractions of compost extract separated by Sephadex G-25.

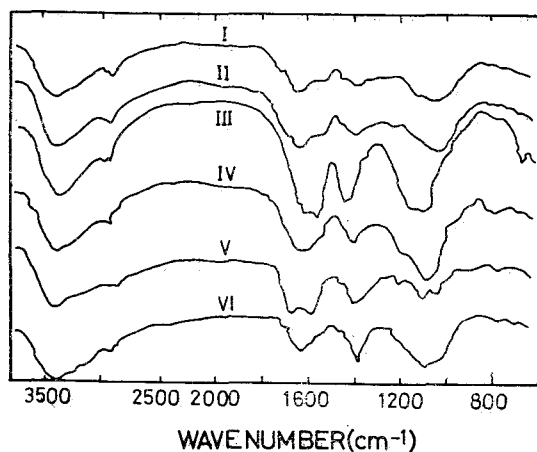


Fig. 9. Infrared spectra of fractions of sewage extract separated by Sephadex G-25.

from different soils, could suggest similar structure of these acids.

A strong absorption band appeared in all spectra at 3400cm^{-1} region. Unassociated OH and NH bonds normally give sharp peaks near 3840 and 3510cm^{-1} , respectively. When association by hydrogen bonding occurs, the bands become broader and frequencies are lowered to 3400 – 3300cm^{-1} . The broad absorption band at 3400cm^{-1} region was, therefore, assigned to alcoholic groups with H bonding. There was some absorption due to NH bonds similarly associated and superimposed on the hydroxyl absorption. Absorption at the 3230cm^{-1} might be due to H-bonded NH stretching vibration^(27,28)

Absorption bands at 2960 , 2925 , and 2850cm^{-1} were identified as methyl and methylene group CH stretching vibrations. Aromatic CH stretching band at 3030cm^{-1} was not recognizable, possibly because of either negligible quantity or a high degree of substitution⁽²⁹⁾.

A strong, broad absorption near 1620cm^{-1} possibly overlapped the carboxylate ion C=O stretching, C=C stretching from aromatic structure, and C=N stretching vibration from the heterocyclic nitrogen compounds⁽²²⁾. Strong absorption at 1400cm^{-1} may be assigned to symmetrical carboxylate C=O bond. The extra high intensity of absorption at 1620cm^{-1} region does not exclude the presence of quinone and nitrogen C=N in cyclic form.

Compost extract showed the strongest absorption in the region of 1400cm^{-1} to which also methyl group deformation is assigned (exactly at 1375cm^{-1}). This suggests the presence of a considerable proportion of methyl groups in the component molecules.

Bellamy,⁽¹⁹⁾ assigned two strong characteristic absorption bands at 1600 , and 1500cm^{-1} to an aromatic ring system. Although absorption in the 1600cm^{-1} region might include C=C stretching of aromatic rings, the absence of significant absorption in the 1500cm^{-1} region with sewage extract suggested that the aromatic compounds were not abundant. However, in case of compost, absorption in the 1500cm^{-1} was strong and broad, possibly due to decomposition products of lignin from garbage.

Sewage extract showed absorption at 1540 , and 650 – 700cm^{-1} , suggesting that it contained abundant polypeptide or protein compounds^(30,31).

Absorption in the 1100cm^{-1} region, so called carbohydrate absorption⁽³²⁾ is usually assigned to C–O–C of sugar ring and glucosidic linkage. According to Cannon⁽³³⁾ and Bellamy⁽¹⁹⁾, OH bending and C–OH stretching vibration was assigned to 1075 – 1000cm^{-1} and glucosidic or sugar ring stretching 1100 – 1170cm^{-1} . In the sewage extract, aliphatic OH and C–OH groups appeared to be responsible for absorption in this region. Absorption by compost extract near 1110

and 1130cm^{-1} , suggests glucosidic linkage and sugar ring vibration.

The infrared spectra of the fractions obtained by Sephadex G-25 (Fig. 8, 9) showed apparently better resolution. Fraction I of compost extract showed absorption in the 1620, 1230, and 1270cm^{-1} is assumed to be due to phenolic or hydroquinone ether C-O-C^(19,33). Possibly the polyphenolic structure might be derived from lignin components of garbage. Since most of the ^{59}Fe was found in fraction I, additional phenolic or quinoid groups may account for its better ability to chelate iron. The other fractions, not associated with ^{59}Fe , still showed strong carboxylate and hydroxyl group absorption. This suggests that these groups in the fractions may not form Fe chelates independently. Schnitzer and Skinner⁽³⁴⁾ also pointed out that carboxylic and phenolic hydroxyls appear to react simultaneously in metal chelation of fulvic acid, but minor types of reactions occur with less acidic carboxyl groups, but alcoholic hydroxyls do not participate in the organo-metallic reactions.

Fraction I, II, and III of the sewage extract showed a significant peak at 1540cm^{-1} possibly overlapped with other carboxylate C=O stretching. Only fraction III showed appreciable absorption at $670\text{--}700\text{cm}^{-1}$ due to NH out of plane deformation of the protein structure. The higher molecular weight fractions I, II, and III, associated with more polypeptide structure, contained most of the added ^{59}Fe . Thus the amide group of peptide linkage in higher molecular weight fractions seemed to provide better chelating ability with Fe.

In conclusion, fraction I of the compost extract, which chelated most of the added ^{59}Fe , showed weak but definite phenolic C-O-C absorption in the 1230 and 1270cm^{-1} regions. This suggests that oxygen groups in polyphenolic structure from garbage compost appear to be effective in Fe chelation. In sewage extract fraction I, II, and III, which contained most of the ^{59}Fe , showed strong definite polypeptide absorption in the region of 1540cm^{-1} due to NH deformation and

C-N stretching of amide groups. These functional groups in these fractions of sewage extract seem to be associated with Fe chelation.

The other fractions, not associated with ^{59}Fe , still showed strong carboxylate carbonyl and hydroxyl groups. Apparently many of these functional groups in the water extracts are not involved in Fe chelation.

Stability Constants of Water-Soluble Chelating Agents

Attempts were made to measure the stability constants of these water-soluble chelating agents by an ion-exchange equilibrium method. Since ferric hydroxide was precipitated even at pH 4.0 it was not possible to measure the concentration of ionic and chelated Fe in solution. However, since the stability constants for Zn chelates from which the strength of chelation with Fe could be predicted were successful the results with Zn were reported here.

As Martell and Calvin⁽³⁵⁾ indicated, the trend of chelation is mostly parallel within the different transition metals with same chelating agents, even though the chelation may depend upon both the properties of chelating agents and those of metals.

The concentration of chelating sites for Zn at pH 4.0 was 0.82 mM for the compost extract and 0.64 mM for the sewage extract. Fig. 10 shows the relationship between relative concentrations of chelating sites and $\lambda_0/\lambda-1$, which are used to determine the stability constants by the ion-exchange equilibrium method. Fig. 10 The stability constant of compost extract at pH 4.0 was 8.23 and that of sewage extract was 9.75. These values are a little higher than those reported by Miller and Ohlrogge (35) for manure extract at pH 6.5.

Clark and Turner (36) pointed out that a plot of $\log(\lambda_0/\lambda-1)$ vs. $\log(\text{Ch})$ is straight only for mononuclear complexes or for limited concentration ranges. They also pointed out that the plot of $\log(\lambda_0/\lambda-1)$ vs. $\log(\text{Ch})$ does not provide suitable means for polynuclear soil organic matter complexes. Probably these values may

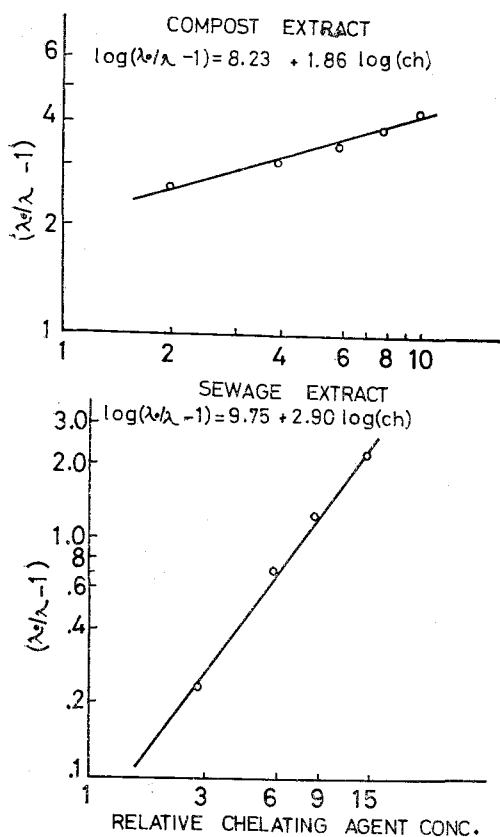


Fig. 10. The relationship used to determine stability constants of water-soluble Zn chelates from solid organic wastes at pH 4.0.

be limited in these concentration ranges and can not be generalized. However they provide approximate estimates of the chelate stability constants.

SUMMARY

This study was conducted to study the properties of the water-soluble natural chelating agents from garbage compost and activated sewage sludge responsible for Fe chelation, which is closely associated with the effectiveness in correcting iron chlorosis in plant. The water-soluble fraction of these materials was fractionated by means of Sephadex gel filtration and the fractions of Fe chelates were traced by radioactive ^{59}Fe . The fractions were examined by ultraviolet and infrared spectroscopy and stability constants for Fe.

The water-soluble fraction from garbage compost was separated by Sephadex G-25 into approximately four fractions.

Most of the added ^{59}Fe was associated with

fraction I, which appeared at the void volume. Further fractionation by Sephadex G-50 indicated that the molecular weight of water-soluble chelating agents is in the approximate range of 5000 to 10,000.

The water-soluble fraction from activated sewage sludge gave six fractions by Sephadex G-25. Most of the added ^{59}Fe was found in the fraction I, II, and III. The molecular weights of most chelating agents associated with ^{59}Fe appeared to be less than 5,000 and those of fraction I that appeared at the void volume was in the range of 5,000 to 1,000.

Discrepancy between radio activity count and UV absorption indicated the heterogeneity of the fractions obtained by Sephadex gel filtration.

Ultraviolet absorption spectra of all fractions separated by Sephadex G-25 and containing chelating agents showed no differences. Fraction IV and V of sewage extract showed absorption maxima and shifting similar to nucleic acid components suggesting the presence of decomposition products of nucleic acid. Similarly fraction VI contained phenolic type amino acid groups.

Fraction I of compost extract contained most of the added ^{59}Fe and showed weak but extra definite absorption in the 1230, and 1270 cm^{-1} region, suggesting that extra oxygen groups in polyphenolic structure were probably involved in Fe chelation.

In sewage extract, fraction I, II, and III in which most of the ^{59}Fe was found, showed strong definite polypeptide absorption in the region of 1540 cm^{-1} due to NH deformation and C-N stretching of amide groups in the peptide-bond. These extra functional groups in fraction I, II, and III appeared to be associated with Fe chelation.

The other fractions, not associated with ^{59}Fe , still have carboxyl and hydroxyl groups, suggesting that these functional groups in these water extracts may not independently form the Fe chelates.

Precipitation of ferric hydroxide precluded measuring the stability constants for Fe-chelates. However, the formation constants for Zn chelates

as log K values for compost extract and sewage extract at pH 4.0 from which the strength of chelation with Fe could be presumed, were 8.23, and 9.75, respectively, indicating strong complexation with metals.

The chelating capacity of compost extract containing 6.5 g organic matter per liter was 0.82 mM, and that of sewage extract containing 5.3 g per liter was 0.64 mM.

LITERATURE CITED

1. Anderson, M.S. : U. S. D. A. Cir. NO 972 (1955).
2. Seiberth, W. : Z. Pflanzenernaehr. Dueng. **91**, 53 (1960).
3. Kick, H. : Trans. Int. Congr. Soil Sci. 7th (Madison, Wis.) **3**, 321 (1960).
4. Miller, B.F., Lindsay, W.L., and Parsa, A. A. : Proc. Conf. Animal Waste management, Cornell Univ., Ithaca P. 120-123 (1969).
5. Parsa, A.A. : Ph. D. Dissertation, Colorado State University, Fort Collins Colo. (1969).
6. Park, N.J. and Lindsay, W.L. : Ph. D. Dissertation, Colorado State University, Fort Collins Colo. (1971).
7. O'connor, G.A., Lindsay, W.L. and Olsen, S.R. : Soil Sci. Soc. Amer. Proc. **35**, 176 (1971).
8. Martell, A.E. and Calvin, M. : Chemistry of metal chelate compounds, Prentice-Hall, Englewood Cliffs. N.J. 1956.
9. Randhawa, N.S. and Broadbent, F.E. : Soil Sci. **99**, 362 (1965).
10. Karpukhin, A.I. and Fokin, A.D. : Izv. Timiryazev. Sel'shokhoz. Akad. NO. 5, 139 (1969) (Abstr. Soils Fert. **33**, 943, (1970).
11. Kaurichev, I.S. and Nozdronova, E.M. : Dokl. S.-kh. Aked. Timiryazeva. **50**, 65 (1960) (Abstr. Soils Fert. **24**: 48, 1961).
12. Smirnova, K.M. and Glebova, G.I. : Pochvo-vedenie NO. **8**, 45 (1958).
13. Gallagher, P.H. : Proc. Roy. Irish Akad. Sect. B. **48**, 213 (1942).
14. Ziechmann, W. and Scholz, H. : Naturwissenschaften **47**, 193 (1960).
15. Beaven, G.H., Holiday, E.R. and Johnson, E.A. : Nucleic Acid, Academic Press, N.Y. 1955.
16. Shugar, D. and Fox, J.J. : Biochem. et Biophys. Acta **9**, 199 (1952).
17. Doty, P. and Geiduschek, E.P. : The Proteins, Academic press, N.Y. (1953).
18. Beaven, G.H. and Holiday, E.R. : Advan. Protein Chem. **7**, 319 (1952).
19. Bellamy, L.J. : The Infrared Spectra of Complex Molecules, 2nd Ed. John Wiley and Sons, Inc., N.Y. 1959.
20. Fraser, R.D.B. Progr. Biophys. Biophys. Chem. **3**, 47 (1953).
21. Kendall, D.N. : Applied Infrared Spectroscopy, Reinhold Publ. Corp. N.Y. 1966.
22. Kononova, M.M. : Soil Organic Matter, Pergamon Press, N.Y. 1966.
23. Sutherland, G.B.B.M. : Discuss. Faraday Soc. NO. **9**, 291 (1950).
24. Goulden, J.D.S. and Jenkinson, D.S. : J. Soil Sci, **10**, 264 (1959).
25. Schnitzer, M., Shearer, D.A. and Wright, J.R. : Soil Sci. **87**, 232 (1959)
26. Kumada, K. and Aizawa, K. : Soil Plant Food **3**, 152 (1958).
27. Mortensen, J.L. and Schwendinger, R.B. : Geochim. et Cosmochim. Acta **27**, 201 (1963)
28. Sutherland, G.B.B.M. : Advan. Protein Chem. **7**, 291 (1952).
29. Cannon, C.G. and Sutherland, G.B.B.M. : Trans. Faraday Soc. **41**, 279 (1945).
30. Beer, M., Sutherland, G.B.B.M., Tanner, F.R.S., Tanner, KN., and Wood, D.L. : Proc. Royal Soc. Ser. A249, 147 (1959).
31. Elliot, A. : Proc. Royal Soc. Ser. A 221, 104 (1954).
32. Farmer, V.C. and Morrison, R.I. : Sci. Proc. Roy. Dublin Soc. Ser. A. **1**, 85 (1960).
33. Cannon, C.G. : Nature (London) **171**, 308 (1953).
34. Schnitzer, M. and Skinner, S.I.M. : Soil Sci. **99**, 278 (1965).
35. Miller, M.H. and Ohlrogge, A.J. : Soil Sci. Soc. Amer. Proc. **22**, 225 (1958).
36. Clark, J.S. and Turner, R.C. : Soil Sci. **107**, 8 (1969).