Chemical composition and molecular weight distribution of dissolved organic matter produced by bacterial degradation of green algae

TOSHIKO AKIYAMA

Department of Chemistry, Tokyo Metropolitan University, Setagaya, Tokyo 158, Japan

(Received March 13, 1972; in revised form November 28, 1972)

Abstract—Chemical composition and molecular weight of dissolved organic matter were determined by employing chemical analysis and Sephadex Gel filtration method with respect to bacterial degradation products of green algae. The chemical composition is as follows; pigment materials 15%, lipid and organic acid materials 30%, proteinous materials 26% and carbohydrate materials 8%. The molecular weight of the dissolved organic matter is distributed in wide ranges. Organic matter with high molecular weight (> 50,000) consists mainly of proteinous and carbohydrate materials, intermediate one (1,500–50,000) consists of pigment and lipid materials. The composition of lower molecular weight matter is not yet known.

The dissolved organic matter produced by bacterial degradation of green algal cells is characterized by yellow colouring, which originates in pigments (in main chlorophyll pigments), and high molecular weight organic substances, which consist almost of proteinous substances with lesser amounts of carbohydrate substances. Although there is a considerable difference between the dissolved organic matter of bacterial degradation products and that in natural water, the former is suggested to be an important source of the latter.

INTRODUCTION

Organic constituents of lake and sea waters have recently become of general interest. The points of this investigation are essentially concerned with geochemical cycle of organic substances and their ecological significance in hydrosphere.

It has been recognized that algae may be important contributors to organic constituents in lake and sea waters. AARONSON (1971) summarized studies on the excretion rate of dissolved organic matter by phytoplankton and biochemical properties of excretion products of algae. Up to now, little is known on the products of bacterial degradation of phytoplankton. SKOPINTSEV et al. (1965) carried out studies on decomposition of the mixtures of phytoplankton and zooplankton, however, little attention was given to the chemical nature of the dissolved organic matter produced
by decomposition. Otsuki (1968) and Otsuki and Hanya (1968) investigated the rate of the production of dissolved organic matter in microbial decomposition of phytoplankton and observed the production of nitrogen-rich dissolved organic matter, which was thought to be proteinous materials. Foree and McCarty (1970) also determined the rate and extent of algal degradation under simulated natural conditions.

In these investigations much attention has been paid to the production rate of dissolved organic matter in excretion and decomposition of phytoplankton.

The purpose of this paper is to investigate the chemical composition and the molecular weight distribution of dissolved organic matter produced by microbial decomposition of dead green algae, and to discuss a possible origin of dissolved organic matter in natural water with special reference to “yellow substances” and high molecular weight substances.

**Materials and Methods**

*Preparation of samples* Microbial decomposition of dead green algae was carried out to prepare samples on the basis of the method of Otsuki (1968). One hundred grams of dried green algae, the *Chlorella* sp., was suspended in a 10 l water containing 45 g of KH₂PO₄ and 120 g of Na₂HPO₄. Water extract from the muds of Lake Haruna was also added to the solution as a source of microflora (consisted mainly of bacteria). The solution was stored in a brown bottle with continuous aeration at room temperature. After 300 days, the solution was centrifuged at 12,000 r.p.m. and the supernatant was filtered through a membrane filter (Millipore filter HA type of 0.45 μm pore size).

![Fig.1. Changes of dissolved organic carbon and nitrogen produced by degradation of algal cells.](image)

- : organic carbon,  ● : organic nitrogen
The changes with time of concentrations of dissolved organic carbon and organic nitrogen in the solution during the storage are shown in Fig. 1. The ratio of dissolved organic carbon to dissolved organic nitrogen in the solution was 5.8 by weight after standing for 300 days.

Group separation of dissolved organic matter

The separation of dissolved organic matter from the aqueous solution was done by a combination of organic solvent extraction, Sephadex gel chromatography and vacuum ultrafiltration through a collodion membrane.

The sample solution was separated into four fractions as follows.

(1) Chloroform fraction: One liter of the sample solution was evaporated nearly to dryness by a rotary evaporator at 45–50°C. The residue was extracted three times each with 100ml portion of methanol. The solvent was removed under reduced pressure. The residue was re-extracted twice with 200ml portions of chloroform. After the chloroform solution was reduced to 2–3ml by evaporation, it was placed on a Sephadex LH-20 column (10 x 450mm) and eluted with chloroform-methanol mixture (2:1). More than 90% of chloroform extract was excluded from the column. The excluded fraction was named chloroform fraction.

(2) Methanol fraction: The residue from chloroform extraction was re-dissolved in methanol. The concentrated solution (4–5ml) was placed on a Sephadex LH-20 column following elution with chloroform-methanol mixture (1:1). More than 82% of methanol-soluble but chloroform-insoluble organic matter was excluded from the column. The excluded fraction was named methanol fraction.

(3) Water fraction 1: Methanol insoluble fraction was redissolved in distilled water. The solution was filtered through a collodion bag (Sartorius membrane-filter Cat-No. sm 13,200). The solution (4–5ml) remaining in the collodion bag was placed on a Sephadex G-50 column and developed with distilled water. The excluded fraction was dried under reduced pressure. The residue was named water fraction 1. More than 90% of dissolved organic matter of the solution remaining in the bag was excluded from G-50 column.

(4) Water fraction 2: The solution filtered through the collodion bag was concentrated (4–5ml) and placed on a Sephadex G-10 column and developed with distilled water. The excluded fraction was dried under reduced pressure. The residue was named water fraction 2. More than 70% of dissolved organic matter passed through the bag was excluded from Sephadex G-10 column.

Chemical tests and reagents

Dissolved organic carbon was determined by the method of MENZEL and VACCARO (1964), Dissolved organic nitrogen by micro-Kjeldahl method, carbohydrate by phenolsulphuric acid method using glucose as a standard, and proteinous material by ninhydrine colorimetric method after hydrolysis with HCl (1:1) for 24 hours, using glycine as a standard. The detection of amino acid was made
by two-dimensional paper chromatography, by using the following solvent systems; phenol : water (75:25) as the first solvent, and n-butanol : acetic acid : water (4:1:1) as the second solvent. Amino acids were detected with 0.2% ninhydrine solution.

Lipids in methanol fraction were separated by both column chromatography and thin-layer chromatography. About 30mg of methanol fraction was placed on a silicagel column (2 × 20cm) and eluted successively with each 250ml portion of n-hexane, benzene, chloroform and methanol. Each effluent was evaporated to dryness and the residue was weighed. Each fraction was developed with thin-layer chromatography on silicagel plates with petroleum ether : ether (4:1) for nonpolar lipids and chloroform : methanol : water (65:25:4) for polar lipids. Lipids were detected by the ammonium phosphomolybdate method.

Phenolic compounds in methanol and chloroform fractions were developed with thin-layer chromatography on silicagel plates and detected with ferric chloride and bis-diazotized benzidine method.

Pigments in chloroform fraction were detected with two-dimensional thin layer chromatography on silicagel plates. The following solvents were used successively; acetone, petroleum ether, and petroleum ether : n-propanol (99:1) in the first step and petroleum ether : chloroform (75:25) in the second step.

Ultraviolet and visible absorption measurements of samples were carried out on a Hitachi EPS-3 Recording Spectrophotometer. Infrared absorption spectra were recorded on a Hitachi EPI Infrared Spectrophotometer by KBr disk method.

**Measurements of molecular weight** Molecular weight distribution was determined by successive filtration through Sephadex gel columns (1 × 45cm columns of G-10, G-15, G-25, G-50, G-75, G-100, G-150 and G-200). Elution was conducted with distilled water. For determination of molecular weight distribution, the material excluded by each gel was collected, and its optical density at 410mµ and 275mµ and organic carbon content were determined.

**Results**

**Molecular weight distribution of dissolved organic matter** The molecular weight distribution of dissolved organic matter produced by bacterial degradation of green algae is shown in Fig.2. As seen in the figure, there are three peaks of the molecular weight in the ranges of 0—700, 5,000—10,000 and 150,000—200,000. The distribution pattern measured by absorbance at 275mµ agreed closely with that measured by organic carbon. But the distribution pattern measured by absorbance at 410mµ somewhat differed from that measured by organic carbon. With respect to the relative abundance of organic matter with molecular weight 5,000—10,000, the amounts of material measured by absorbance at 410mµ is higher than those of materials by organic carbon and absorbance at 275mµ. This shows that the materials with molecular weight
from 5,000 to 10,000 are more highly coloured than those of the others. Although it is difficult to assign accurate molecular weight to each fraction, the figure shows the general pattern of molecular weight distribution in dissolved organic matter under neutral pH.

**Chemical composition of the fraction** The result of analysis of each fraction was summarized in Table 1. The absorption spectra of ultraviolet, visible and infrared regions were shown in Fig.3 and Fig.4, respectively.

Table 1. Elemental analysis of the fractions

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Ash %</th>
<th>Elemental composition ash free %</th>
<th>Molecular weight</th>
<th>Absorbance per mg/ml</th>
<th>Percent to total org.C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform frac. (pigment)</td>
<td>0.98</td>
<td>7.89 60.13 3.96</td>
<td>1,500–50,000</td>
<td>10.0 6.97</td>
<td>15.5</td>
</tr>
<tr>
<td>Methanol frac. (lipid and organic acid)</td>
<td>1.10</td>
<td>6.73 52.60 6.78</td>
<td>1,500–50,000</td>
<td>8.93 1.42</td>
<td>36.4</td>
</tr>
<tr>
<td>Water frac. 1 (high-mol. wt. proteinaceous subs.)</td>
<td>1.45</td>
<td>7.29 49.66 10.87</td>
<td>50,000–200,000</td>
<td>2.22 0.832</td>
<td>25.4</td>
</tr>
<tr>
<td>Water frac. 2 (carbohydrate and proteinaceous subs.)</td>
<td>5.81</td>
<td>6.41 44.41 5.40</td>
<td>700–50,000</td>
<td>2.09 0.711</td>
<td>5.6</td>
</tr>
</tbody>
</table>
Fig. 3. Ultra- and visible absorption spectra of dissolved organic matter. 1: chloroform fraction of fresh cells, 2: chloroform fraction of DOM, 3: methanol fraction of DOM, 5: water 2 fraction of DOM.

Fig. 4. Infrared spectra of dissolved organic matter. 1: chloroform fraction, 2: methanol fraction, 3: water 1 fraction, 4: water 2 fraction.
Chloroform fraction (pigment fraction)

As seen in Fig. 3, the maximum absorption peaks of chloroform fraction were observed at 410, 508, 540, 610 and 670 μm, which were in good agreement with those of phaeophytin a. The characteristics of infrared absorptions, as shown in Fig. 4, in the regions of 1,750–1,600 cm⁻¹ (carbonyl), 1,460–1,360 cm⁻¹ (CH bending) and 1,300–650 cm⁻¹ (finger print region) also indicate the presence of chlorophyll pigment. From the comparison of the infrared spectra of dissolved organic matter of the residual algal cells with those of the fresh algal cells, it seems that chlorophyll structure still remained through the degradation process. The result of thin layer chromatography showed that this fraction consists mainly of chlorophyll pigment, and that carotenoid and xanthophyll pigments were not detected although large amounts of them were found in the chloroform extract of fresh algal cells.

From these results, it is concluded that the chloroform fraction mainly consists of chlorophyll pigments, and consequently this fraction is designated as “pigment fraction” in the present paper.

Methanol fraction (lipid fraction)

Methanol fraction did not show any characteristic absorption, but has two discernible shoulders at 280 and 410 μm. As seen in Fig. 4, the spectrum of methanol fraction showed strong absorption in the region of 1,750–1,700 cm⁻¹ which is attributed to carbonyl groups. The absorption peaks of CH₂ in the region of 1,460–1,100 cm⁻¹ (1,460, 1,370, 1,260 and 1,160) and the strong absorption peak of carbonyl at 1,730 cm⁻¹ agree with the peaks of lipid compounds which are characterized by long chains of -CH₂- and -C- or -C-O-. The chromatographic nature of lipid substances is

| Table 2. Chromatographic nature of methanol (lipid and organic acid) fraction; 31.0 mg of the fraction was applied |
|---------------------------------|--------|-----------------|
| Fraction | Weight mg | Rf values of TLC |
| n-Hexane frac. | 0 | 0 |
| Benzene frac. | 3.0 | 9.7 |
| Chloroform frac. | 14.3 | 45.9 |
| Methanol frac. | 10.4 | 33.2 |
| Loss | 11.2 |
| a. petroleum ether:ether (4:1) | b. chloroform:methanol:water (65:25:4) | c. toluene:ethylformate:formic acid (5:4:1) detected by the ferric chloride and the bis-diazotized reagent for phenolic compounds |
shown in Table 2. Any hydrocarbon (hexane eluted) was not detected, but seven kinds of aromatic lipids (benzene eluted) and five kinds of simple lipids (chloroform eluted) were found chromatographically in this fraction. Methanol eluted as much as 30% of this fraction and five kinds of complexed lipids were found. Phenolic compound was not detected.

It is concluded that the methanol fraction consists mainly of lipid materials and this is designated as “lipid fraction”.

Water fractions 1 and 2. (carbohydrate and proteinous fractions)

Both water fractions 1 and 2 did not show any characteristic absorption peak in visible and ultraviolet regions (Fig.3). On the other hand, the infrared spectrum of the water fraction 1 is similar to that of proteinous matter (3,300–2,860 cm⁻¹ and 1,660–1,540 cm⁻¹). The elemental composition also shows that the water fraction 1 has high nitrogen content. The infrared spectrum of the water fraction 2 also shows the presence of proteinous materials. Content of carbohydrate, composition and content of amino acid in hydrolysate of the water fractions are summarized in Table 3. More than 90% of the water fraction 1 consists of proteinous material. In the water fraction 2 amino acid content is not so high as in the water fraction 1, but carbohydrate content of the water fraction 2 is higher than that of the water fraction 1. The results of analysis of the hydrolysates show the presence of 11 kinds of amino acids for the water fraction 1, and 9 kinds of amino acids for the water fraction 2.

From these results, it is concluded that the water fraction 1 consists mainly of proteinous materials with higher molecular weight, and the water fraction 2 consists mainly of the mixture of proteinous and carbohydrate materials with lower molecular weight. Thus, these two fractions are named “proteinous fraction” and “proteinous and carbohydrate fraction”, respectively.

**DISCUSSION**

The relation of the chemical composition with the molecular weight distribution...
of dissolved organic matter produced by bacterial degradation of green algae is given in Fig.5. It is to be noticed that the dissolved organic matter of high molecular weight consists of large amounts of proteinous material and small amounts of carbohydrate. The dissolved organic matter of intermediate molecular weight consists mainly of pigment and lipid materials. These organic matters of relatively high molecular weight occupy more than 80% of total dissolved organic matter. Although the dissolved organic matter of low molecular weight was not studied in detail, they may consist of free amino acids, organic acids, free sugars etc.

Nature and origin of yellow substances in natural water have been discussed by many workers (for example, KALLE, 1937; Fogg and Boalch, 1958; KhaIlov, 1962), and they suggested that the substances originated from algal materials in water. The present result also confirmed the previous works. The extent of contributions to yellow colouration due to each organic fraction was estimated on the basis of the absorbance at 410 m\(\mu\); pigment 60%, lipid 30% and proteinous + carbohydrate 10%. Thus yellow colouration of dissolved organic matter in the degradation of green algae is supposed to have resulted chiefly from pigment and lipid materials. In the degradation process of green algae, chlorophyll pigment remained almost in an unaltered form. Even though lipid fraction is highly coloured, most of yellow colour may be caused by the colour of chlorophyll degradative products and chlorophyll pigments probably bound to lipid. Ghassemi and Christman (1968) reported that the colour producing molecules in natural water consist mainly of materials with apparent molecular weight from 700 to 10,000. The molecular weight distribution and the chemical and spectro-
scopic natures of yellow organic substances studied by Shapiro (1957) are similar to those of the lipid fraction of the present work. Sieburth (1969) found considerable amounts of phenolic compounds in exudate of brown algae and pointed out the importance of phenolic compounds in the formation of “Gelbstoff” in sea water, but any phenolic compound was not detected in the present study on the bacterial degradation products of green algae. The colouration of proteinous fraction of high molecular weight is not so strong as that of pigment and lipid fractions. The weak colour of the fraction seems to be due to the traces of pigment mixed or combined with proteinous and carbohydrate substances. From these results it is concluded that most of yellow coloured materials in dissolved organic matter produced from algae originates from chlorophyll pigments of algal cells.

The molecular weight distribution pattern of dissolved organic matter in freshwater samples was reported by Gjessing (1966) and Gjessing and Lee (1967). The concentration (about 25%) of high molecular weight organic matter (> 50,000) of dissolved organic matter in this study is higher than that reported by them. With respect to the composition of high molecular weight organic matter, it consists mainly of proteinous and carbohydrate materials in this study, but this result is much differed from the results of Khailov et al. (1969). He reported that the high molecular weight organic substances in sea water contained only small amounts of protein (1.6—7.4%) and carbohydrate was also low (1.6—23%), and that most of high molecular weight organic substances in sea water were thought to be polymers without protein and carbohydrate.

It has been already pointed out by Otsuki (1968) that dissolved organic matter produced by bacterial decomposition of algae was rich in proteinous material. The present result confirmed that most of nitrogen in the degradation products of green algae exist as proteinous materials of high molecular weight. Extracellular products of algae (e.g. Watt, 1966) and exudates of algae (Sieburth, 1969) are higher in carbohydrate content and lower in protein content compared with those in this study. But Aaronson (1971) found that phytoflagellates secreted considerable amounts of protein of macromolecule. The ratio of protein and carbohydrate to total dissolved organic matter is not so high in sea water (Duursma, 1965), and the ratio of protein to carbohydrate is lower than that of the bacterial degradation products. For example, the concentration of dissolved proteinous substances was 30—100µg/l in Pacific Ocean (Kawahara and Maita, 1971) and that of “combined amino acids” was 2—120µg/l in the Irish Sea (Riley and Segar, 1970), and that of dissolved carbohydrate was 0.09—0.46mg/l in the Kuroshio (Handa, 1967) and 1.5—3.3mg/l in lake water (Walsh, 1966). The ratio of lipid to total dissolved organic matter in natural water is also lower than that of the bacterial degradation products. The concentration of lipid in sea waters in Gulf of Mexico ranges from 0.17 to 1.59mg/l and chloroform extract (lipids) contains 10 to 20% of the total dissolved organic carbon (Jeffrey, 1970). It is to be
noticed that the dissolved organic matter produced by bacterial degradation of phytoplankton cells can be characterized by high concentrations of proteinous substances and lipids and relatively lower concentrations of carbohydrates, and the chemical composition of it is rather similar to that of algal cell itself (Olive and Morrison, 1967). Therefore, it is assumed that bacterial degradation produces dissolved organic matter of which composition is very similar to that of algal cells.

These discrepancies between the nature of organic matter produced by bacterial degradation and dissolved organic matter occurring in natural water can be explained as follows: High molecular weight proteinous substances produced by bacterial degradation may be in part altered to particulate form and utilized by organisms and in part altered to more stable and more complex substances which are resistant to biological attack, and the residual dissolved organic matter after utilization or alteration into particulate form may be a source of dissolved organic matter in natural water.

CONCLUSION

It was elucidated that dissolved organic matter produced by bacterial degradation differed from dissolved organic matter in natural waters in some respects. From these results, dissolved organic matter which was produced primarily by bacterial degradation was suggested to have been secondarily subjected to biological and chemical changes or fractional precipitation and to have changed to more stable form.

It was recognized that investigations on the behaviour of these bacterial degradation products in natural waters are important in geochemical aspects.

ACKNOWLEDGMENTS

The author thanks Prof. T. Hanya, Tokyo Metropolitan University, for his valuable advice and encouragement, and also thanks Dr. N. Ogura, Tokyo Metropolitan University, for his helpful discussions.

REFERENCES

Gjessing, E. T. (1966) Humic substances in natural water: Method for separation and characteri-


