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J. Leyendekkers

Fluorophors and Light-absorbing Substances in Natural Waters

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INTRODUCTORY NOTE

This report was compiled December 1966 by Mrs.J. Leyendekkers at the close of her ten months stay with the Royal Netherlands Meteorological Institute, section of oceanography. The bulk of the report consists of a rather comprehensive literature review but a short account of a few simple measurements made by the author are included, as well as a number of valuable suggestions for further research (see part IV: Discussion).

The author was reluctant to submit her report for publication in one of the current scientific journals since she thought there were too many shortcomings and gaps in her review. Therefore her report has been included in the series of the Institute's "Wetenschappelijke Rapporten" (Scientific reports) so as to be able to distribute it on a restricted scale to interested laboratories and persons.

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Prof.dr.W. Bleeker.
Director-in-Chief

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FLUOROPHORS AND LIGHT-ABSORBING SUBSTANCES IN NATURAL WATERS

This report reviews some of the investigations of the ultraviolet- (UV-) and visible-light-absorbing substances and fluorescent substances commonly present in fresh water and in seawater.

The chloroplastic pigments have been most thoroughly studied and their spectral and chemical characteristics are known. The yellow organic acids in lake water have also been rather fully characterised but their exact structure has not yet been determined. Information regarding other such substances in natural waters is restricted to their spectral characteristics and their probable origin and mode of formation. Nevertheless, the optical properties of these substances have been the basis of a number of studies of water structure and movement. A brief review of these studies is given. Investigations concerned with relationships between the light-absorbing substances and fluorophors and the biochemical cycle are also reviewed.
Some possibilities for future research are considered.

I. Light-absorbing Substances

(a) STABLE ORGANIC SUBSTANCES

The work of a number of authors indicates that there are two main groups of fairly stable organic compounds dissolved in natural waters. The first group derives from land drainage while the second group derives from the decomposition of organisms in the water (e.g. plankton, bacteria).

Birge and Juday (1934) grouped data from several hundred lakes and distinguished between two compounds:

(1) (allochthonous) derived from bogs, peat and soil and having a brown colour and an approximate C/N ratio of 45-50 and
(2) (autochthonous) derived from the decomposition of plankton and having an approximate ratio of 12.

Kalle (1961) defined his "Gelbstoff" (first discussed by him in 1937) as a mixture of different coloured organic compounds dissolved in all natural waters and distinguished between:

(1) the phenol-humic acids (pyrogallol-humic acid and hydrochinon-humic acids) which are light to dark brown (presumable in solution) and the dominant compounds in fresh water, and
(2) the carbohydrate-humic acids or the melanoidines which are light to golden yellow, are more stable than the phenol-humic acids and the dominant "Gelbstoff" compounds in seawater. They derive from the breakdown of organic matter.

Skopintsev (1959) refers to a stable complex of a carbon-protein type (possibly a pectin or uron) that is present in seawater and derives from the decomposition of dead organisms. This complex (which has a C/N ratio of 10) has similar properties to what Skopintsev describes as "lake humus of the eutrophic and the oligotrophic types" but differs from the "water humus of coloured waters derived from water-soluble compounds in soil humus".

Gorham (1957), from his study of 23 Nova Scotian lakes (including both clear and peaty waters), concluded that one group of dissolved organic substances derived from the soil or peat and another group from the aquatic organisms; the former substances were in much higher concentration.

Shapiro (1957) extracted and studied the dissolved coloured organic compounds in a number of Ontario lakes and concluded that they were derived from the land and not from the organisms in the lakes. On the other hand, Fogg and Boalch (1958) demonstrated that yellow organic compounds were produced in filtrates of mass cultures of marine algae: in filtrates of old cultures considerable concentrations of such compounds occur.

The organic substances derived from the land will be referred to as Group I and those derived from organisms in the water as Group II.

Group I

Optical properties:
Shapiro (1957) measured the absorption of his organic acids from 700mu to 300mu [1] and found only slight absorption at wavelengths greater than 450mu, but strong absorption for shorter wavelengths, especially those near 300mu. Gorham (1957) obtained similar absorption spectra for his "soil-derived" organic substances. Kalle (1961) measured the absorption of his phenolic humic acids in the range 387 to 665mu and found that they absorbed more strongly in the blue than the red although the selectivity was not as great as that shown by the carbohydrate humic acids. Jerlov (1957) measured the absorption of brown Swedish lake water mixed with salt water (the sample was filtered after 6 months and absorption then measured) and compared it with the absorption of water from Gullmar Fjord (concentrated and filtered). The curves were very similar and showed almost a logarithmic increase towards shorter wavelengths (absorption increased rapidly from 400 to 260mu).
Shapiro's acids showed a yellow-green fluorescence, on paper and in solution, when excited by UV light (? wavelength used).
Gorham did not mention any fluorescence. Kalle (1963) found the fluorescence of bog water (which contains this group) very high but did not specify the spectral peak.

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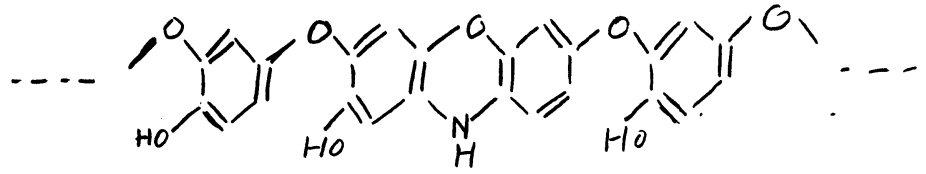
1) In this report "mu" should be always read as "mμ" (10^{-9} metre); "ug" should be read as "μg".

Chemical properties:

Since Shapiro's acids are probably related to the water humus derived from the soil (that Skopintsev refers to) and the phenolhumic acids of Kalle it is assumed that the properties of Shapiro's acids are fairly representative of this group.

The dried ethyl-acetate extracts form a deep orange film which becomes brown when ground to a powder. In solution the colour ranges from light yellow to dark brown depending on the concentration. The intensity of the colour increases with increasing pH - this colour change is reversible. The colour is bleached by sunlight and is lost when the acids are oxidised under acid conditions. The free acids are moderately water soluble, very soluble in basic solution but insoluble in nonpolar solvents. The salts are extremely water soluble and are soluble in aqueous alcohol but in no other commonly used solvents.
Melting point: 95-150°C
Average molecular weight: 456
Empirical formula: ($C_{50}H_{62}O_{27}N$). Shapiro believed the N to be an impurity but from the work of Swain, Blumentals and Millers (1959) it seems likely that the nitrogen derived from amino acids linked to the organic acids in some way. Thiele and Kettner (1953) visualised that the nitrogen might occur in the humic acid molecule as a bridge substance of the nature of oxazine:



This may also be the way in which the amino acids are incorporated into the humic acid micelle. The above empirical formula gives a C/N ratio of about 50, in agreement with that proposed by Birge and Juday for the allochthonous substances from bogs, peat and soil. Shapiro's acids were also able to retain iron in a filterable state at pH values up to 13.2 (in control solutions the iron rapidly precipitated out)*
The acids had a delicate fruity odour.
(for more details see Shapiro's paper of 1957).

Shapiro concluded that they were hydroxy carboxylic acids, probably phenolic or enolic in character.

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* the solubilising effect of these acids on iron is a particulary interesting property and suggests chelation. The present author found that the fluorescence of pond water and river water was quenched by F^{2+} and Fe^{3+}. D. Eisma (personal communication) added these ions to river water, estuarine water and seawater and concluded that easily chelating fluorophors only occur in fresh water.

Group II

Optical properties:

Kalle (1961) gives the spectral absorption of the melanoidines in the range 665-387mu. The characteristic feature is a very strong selective absorption in the blue end of the spectrum. This has been substantiated by other workers, who extended the measurements to the far UV. However, the spectral characteristics of this group remain only broadly defined, the details no doubt obscured by superimposed spectra of a variety of other substances.

One of the most striking features of the UV and visible absorption spectrum of the algal substances investigated by Fogg and Boalch (1958) was the peak at 265mu. The yellow substance extracted by Chanu (1959) from coastal water showed a maximum absorption at about 250mu.

Armstrong and Boalch (1961) measured the absorption (200-400mu) of filtered seawater and seawater culture solutions in which algae had been grown. Absorption was high from 300-200mu, especially for water with a heavy diatom bloom (from the Firth of Clyde) and culture solutions.

Sournia (1965) has briefly discussed the work done on absorption of visible and ultraviolet light by natural waters (1927 onwards) and has measured the UV absorption in the coastal waters of Nossi Bé (Madagascar) during March and June 1965. From Sournia's results and previous work the most significant and characteristic absorption for natural waters occurs at the wavelengths 220, 250, 265, 300 and 350mu and of less importance 330 and 370mu. The peak around 265mu is attributed to the double bonds of purine pyrimidine bases, although maximum absorption for certain amino acids is near this wavelengths, e.g. tyrosine. The water studied by Sournia contained substances from the plankton and the land and so his spectra contain contributions from both Group I and II, however, water from the most landward station showed a much stronger absorption at 240mu than water from the other stations.

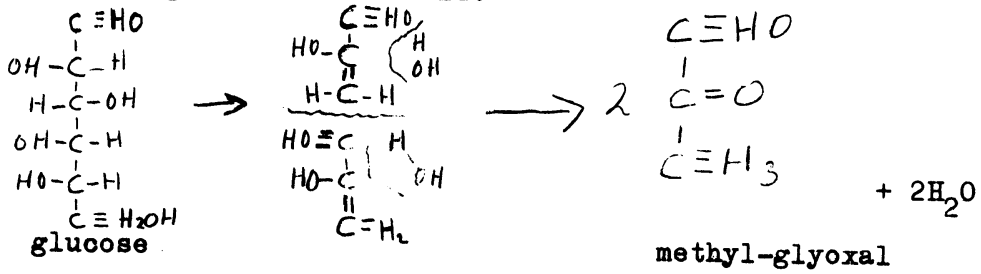
This group is associated with a "blue" fluorophor which is yellow coloured in solution and yellow-orange in the solid state.

Chemical properties:

Although a number of workers (e.g. Kalle (1937) and on), Chanu (1959, Johnston (1955), Ogura (1965)) have extracted "yellow substances"(YS) from seawater little information is available on their chemical properties. However, it seems generally agreed that the C/N ratio for this group is around 10-12.

Kalle (1949) refers to the melanoidin "Gelbstoff" as a "humuslike substance of yellow colour and of comparatively low molecular weight as illustrated by the ease with which dialysis takes place". According to Kalle (1961) the melanoidines are easily formed wherever free carbohydrates and free amino acids are present, i.e. where organisms are disintegrating (e.g.

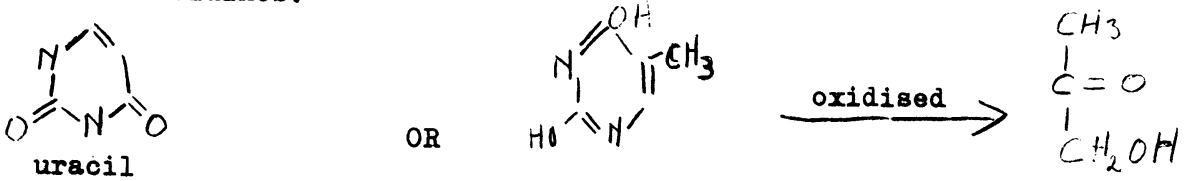
plankton or bacteria). The breakdown of the carbohydrate yields methyl-glyoxal which is readily condensed into a high-molecular yellow resin. The reaction is greatly intensified by the presence of amino acids or in the precense of alkali.



glucose

methyl-glyoxal

It is possible that certain biological substances such as isoleucine and uracil (which Belser (1959) found to be frequently present in coastal water (off Scripps' Pier)), also break down to simple, reactive substances like methyl-glyoxal or substances related to acetol such as acetylmethyl carbinol, acetolhydroxy-butyric acid, etc. and thence form the melanoidines.



uracil
2,4(1,3)-pyrimidinedione

OR

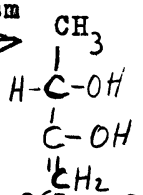
thymine (a pyrimidine)

oxidised

acetol

valine, leucine and isoleucine

metabolism



acetylmethyl carbinol, etc.

As mentioned above the absorption peak at 265mu found for algal yellow substances suggests the presence of purines and pyrimidines or amino acids in high concentration: Fogg and Boalch (1958) confirmed the highly nitrogenous nature of the algal compounds they extracted.

Yentsch and Reichert (1962) investigated the relationship between the pigments of the chloroplasts and the yellow, UV absorbing substances. They found that there was an inverse relationship between the appearance of the yellow substances in the filtrate and the decomposition of the chloroplastic pigments. Yentsch and Reichert interpreted this as indicating that the yellow, UV absorbing substances were initially components of the pigment complex, specifically the protein carrier. This protein carrier has been isolated for the precursor pigment protochlorophyll and the UV absorption of this holochrome is identical in most respects to algal yellow substance. Apparently, the carrier may be one or more amino acid residues such as tyrosine: also nucleic acids or their hydrolysis products may be involved. Yentsch and Reichert concluded that the yellow compounds appear in filtrates only as the result of cell disintegration, thus accounting for the increased concentration of yellow materials as the culture ages. They add, that some portion of the water-soluble substances is readily utilesd as bacterial substrate (whether it is the coloured substances or not is uncertain).

(b) PLANKTON PIGMENTS and OTHER ABSORBING SUBSTANCES

Apart from the YS which seem to have their maximum absorption in the UV region the other common group of absorbing substances are the chlorophylls and related pigments.

The major absorption peaks for the chlorophylls are around 680mu and 410mu. Tyler (1964) found an absorption peak at 515mu for his in situ spectrographic measurements for chlorophyll and other pigments in coastal waters. He did not find this peak in the parallel measurements that he made on fresh-water plants in a Southern Californian pond. He thought this peak might be due to organic substances derived from a group of marine phytoplankton not represented in fresh water.

Absorption characteristics of acetone extracts of some of the various pigments are:

chlorophyll a — 675mu and 410-440mu are major peak regions, a minor band occurs at 620mu

chlorophyll b — this pigment gives increased absorption from 550mu to the shoulder of the chlorophyll a peak at 675mu

chlorophyll c — the major peak occurs around 630mu

chlorophyll d — " " 710mu

xanthophyll absorbs between 450-480mu

phycoerythrin has a major peak at 620mu (in aqueous solution this peak is at 550-570mu. Another water-soluble phycobilin pigment, phycocyanin, absorbs at 630mu in aqueous solution)

cytochrone c — (characteristic for the marine bacterium Nitrosomonas) has three separate bands at 415mu, 521mu and 550mu respectively.

The carotenoids are apparently the most stable components of the pigment system (Yentsch and Ryther, 1959) and show strong absorption in the region 400-500mu. One of the commonest components of carbon extracts of seawater by Johnston (1959) was a coloured material which he believed to be carotenoid.

Van Norman et al (1948) examined the absorption and fluorescence spectra of two marine red algae. The water soluble pigments extracted from one of the algae (Iridaea) showed maximum absorption at 329mu (this absorption was ten times that shown by the pigments absorbing in the visible region).

Ferric salts absorb in the blue spectral region but their concentration will in general be too low to give appreciable absorption. Absorption below 240mu can however be largely attributed to certain inorganic salts. The results of Ogura and Hanya (1966) show that the absorption in the range 210-230mu for filtered seawater was almost entirely due to bromide and nitrate and to a smaller extent by organic matter.

Finally, it is interesting to note that the commonest components of carbon extracts of seawater found by Johnston (1955) were: (1) an insoluble white substance, (2) coloured material that was probably carotenoid (as mentioned above) (3) a fairly large proportion of brownish waxy of fatty matter (? melanoidines) and (4) yellowish orange matter with a bright blue fluorescence in UV. Earlier, Wilson and Armstrong (1952) had found the two most common components of the organic matter extracted from coastal and estuarine water to be a waxy greenish yellow solid with feathery brownish crystals and a whitish opaque matter.

II. Fluorophors

(a) FLUOROPHORS ASSOCIATED WITH YELLOW SUBSTANCE (YS)

The investigations of Kalle (1937 and onwards) indicate that the most commonly occuring group of fluorescent substances in natural waters is characterised by emission in the spectral range described as "blue" (the peak emission is apparently around 400-420mu). This group of substances is derived from the breakdown of organic matter, via reactions parallel to but independent of those which lead to the formation of the carbohydrate-humic acids referred to in the previous section. Kalle (1959) distinguishes between this "free fluorescence" which is widely distributed on the earth's surface and the "blue" fluorescence associated with the breakdown of chlorophyll. This latter fluorescence was emitted by acetone extracts of particulate matter filtered from seawater (Kalle, 1951), and was assumed by Kalle to derive from an organic compound of bacterial origin. The intensity of the fluorescence was found to be reciprocal to the concentration of chlorophyll.

Kalle does not distinguish between the fluorescence associated with the carbohydrate-humic acids,and that associated with the phenoloc-humic acids which are te major "Gelbstoff" substances in fresh water; the fluorescence of bog water, which is rich in these latter substances, is very high (Kalle, 1963). On the other hand, Shapiro (1957) distinguishes between his yellow organic acids and Kalle's carbohydrate-humic acids partly on the basis of the difference in emission spectra of the two groups of substances. Shapiro's acids had a "yellow-green" fluorescence. Shapiro briefly mentions a substance from Long Island Sound which he assumed to be similar to Kalle's carbohydrate-humic acids and thus, although he did not specify the colour, the fluorescence of this substance was probably "blue".

It is interesting to note that when pondwater and river water were excited at 360mu the dominant fluorescence peaks were at 400-405mu (blue) and 550-580mu (yellow-green)*: these measurements were carried out by Dr. Van der Maas of the University of Utrecht, on sample stores for approximately 4 weeks. The intensity of the "blue" peak and other peak were in the ratio 7:3 but the excitation for the latter was certainly not optimal and the intensities would probably have been comparable if this had been so.

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* all the wavelengths quoted in this paper are "uncorrected" (see Udenfriend 1964) which makes comparisons difficult.

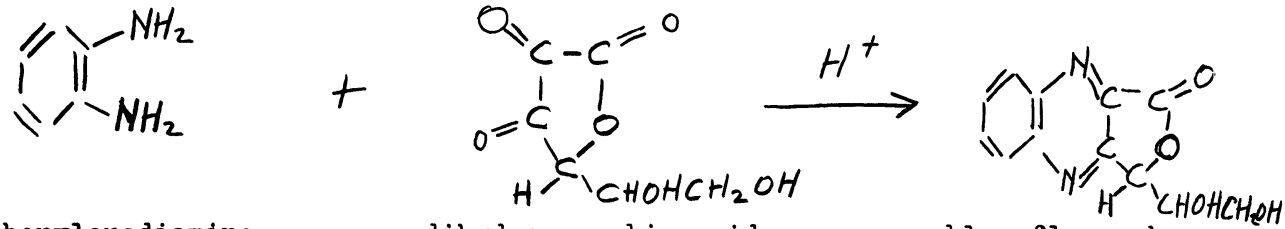
One of the most common components of the organic matter extracted from seawater by Johnston (1955) was a yellow orange matter with a bright blue fluorescence under excitation in the UV. Johnston thought that this substance was probably the blue fluorophor of Kalle (? that from the chlorophyll breakdown or the "free" fluorophor).

Identity

In 1963 Kalle showed that fluorescent substances (with remarkably similar properties to those of the "free" fluorescence" substances) could be formed from carbohydrates, either alone or in the presence of amino acids. The fluorescence yield is generally much higher when amino acids are present although some nitrogen compounds seem to inhibit the reaction when no alkali is added. Kalle was not able to separate and identify these substances but concluded that there were two basic substances formed, both ring structures but one containing N. The N-containing substance was rapidly destroyed by boiling with potassium persuflate in neutral or acid solution, whilst the other substance was not affected. On the other hand, the fluorescence of the N-containing substance did not diminish appreciably when the pH was reduced to give acid conditions whilst that of the other compound was reduced to about 30% of its initial value.

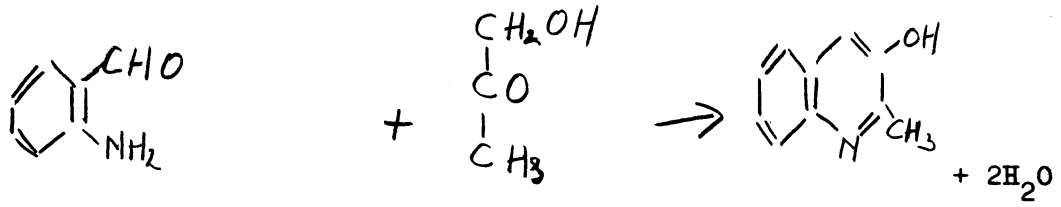
Yentsch and Reichert (1962) do not mention any fluorescence associated with the yellow, UV-absorbing substances of algal filtrates (the protein carrier). However, Laland et al (1966) have investigated a blue fluorophor liberated from certain protein fractions (polypeptides from bovine liver and lipo-protein from human plasma). This fluorophor has major absorption peaks at 250mu and 360mu.

Towne and Spikner (1963) found that a variety of carbohydrates react with o-phenylenediamine in strong acid solutions to form stable, fluorescent substances which were presumed to be substituted quinoxalines. The fluorescent substances obtained from numerous sugars possessed approximately the same excitation and fluorescence wavelength maxima (360mu and 460mu respectively), the maxima for dihydroxyacetone and DL-glyceraldehyde were 335mu and 435mu. It is possible then that the N-containing fluorescent substance of Kalle is a substituted quinoxaline, perhaps something like the blue fluorophor formed by the reaction of o-phenylene-diamine and dihydroascorbic acid under acid conditions as reported by Udenfriend (1964)



o-phenylenediamine dihydroascorbic acid blue fluorophor

On the other hand, acetol, pyruvic acid, dihydroxyacetone, glyceraldehyde, glycolaldehyde and acetaldehyde react with o-aminobenzaldehyde to form hydroxyquinolines, which exhibit blue fluorescence, e.g.



o-aminobenzaldehyde acetol 3-hydroxyquinaldine (2-methyl, 3-hydroxyquinoline) excitation: 365mu, Fl.400-440mu

(N.B. glucose $\xrightarrow[\Delta]{\text{alkali}}$ acetol)

A great deal of research has been carried out on the browning of milk which involves the formation of melanoidin and is associated with the formation of fluorescence substances. Patton (1952) heated lactose with N compounds (e.g. glycine and lysine) and without N compounds. The formation of furan compounds was independent of the presence of N, but maltol was only formed when N was present. Kalle's fluorescent compound without N might therefore be a furan derivative. Maltol is probably an intermediate in the reactions that lead to the formation of the N-containing fluorophor.

Apart from these speculations no information on the identity of Kalle's fluorophors can be offered and so they must be classified as unknown.

Chemical properties

According to Kalle (1949) the "blue" fluorophors are very soluble in water, low molecular weight alcohols and in acetone but insoluble or only slightly soluble in other commonly used organic solvents. They are yellow-coloured in solution (when in sufficiently high concentration) and yellow-orange in the solid state (Johnston, 1955). The effects of pH and chemical stability have been mentioned above.

Preliminary experiments of the author showed that the "blue" fluorescence of decomposed sugar solutions was not quenched with Fe^{2+} whereas that of pond water was. D. Eisma (personal communication) found that the fluorescence of fresh water was quenched by Fe^{2+} but that of seawater was not. The author found that the rate of quenching by ferric ion (Fe^{3+}) was the same for decomposed carbohydrate solutions and a sample of seawater from the Southern Bight (freshly collected). The rate for pond water was much higher.

As mentioned previously Shapiro considered the organic substances he studied to be hydroxycarboxylic acids but their structure is not known. Their properties have been listed in part I.

(b) OTHER BLUE FLUOROPHORS

Extracted from natural waters

Zechmeister and Koe (1952) obtained a number of highly fluorescent aromatic polycyclic hydrocarbons from barnacle extracts (anthracene, phenanthrene, chrysene, fluoranthene, 1,12-benzperylene and 3,3-benzpyrene). They believed that these compounds originated from tarry materials (including detritus) floating along the Southern California coast, e.g. genuine marine tars, coal tar coatings of ships, etc. The fluorescence of these substances ranged from 360mu to 450mu in various organic solvents and in sulphuric acid and was comparable or greater than the fluorescence of quinine (the fluorescence peaks in water were not given).

Bourcart and Mallet (1965) found fairly high concentrations of benzo-3,4 pyrene type polybenzenic hydrocarbons in the central region of the Tyrrhenian Sea (Naples Bight). They assumed that these compounds derived from the waste of the numerous factories in the area (blast-furnaces, cement factories, chemical factories, etc.) and also from land runoff (area has volcanic soils which are still very unstable). These compounds are fluorescent - benzo-3,4 pyrene has a fluorescence 13 times the intensity of a comparable concentration of quinine (wavelengths: in pentane: abs.381mu, fl.403mu; in sulphuric acid: abs. peaks at 385, 470, 493 and 525, fl. peak at 548mu). It is of interest that benz(a)pyrene is oxidised by UV light to benzo(a)pyrene quinones (Masuda and Kuratsune, 1966).

Likely to be present in natural waters

Apart from the blue fluorophors already mentioned there are a number of substances with blue fluorescence that are derived from plant and animal metabolism and which are likely to be present in natural waters at certain times. There are also other substances, some already extracted from natural waters, which can react with each other to form blue fluorophors.

The catabolism of the amino acid trytophan (which has been isolated from both fresh and seawater), catalyzed by the enzyme tryptophan pyrrolase, leads to a large number of substituted aminophenols and quinolines most of which are highly fluorescent with peaks ranging from 405mu to 460mu.

The pyrimidine compound thymine is oxidised to acetol which can react with certain N compounds to form blue fluorophors (e.g. with o-aminobenzaldehyde it forms 3-hydroxyquinaldine, which fluoresces in the range 400-440). Uracil and isoleucine, which commonly occur in both fresh and seawater, release acetol or related compounds and can therefore also give rise to blue fluorophors under the right conditions.

Many of the Kreb's cycle intermediates (malic acid, citric acid) can react with phenols to give substances with blue fluorescence, e.g. the polycarboxylic acids react with resorcinol or orcinol to give fluorescein or umbelliferone derivatives most of which emit a blue fluorescence. A number of these acids have been extracted from fresh and seawater.

Various pterins from fish exhibit "blue" fluorescence. As well, a large number of the substances derived from plants have a blue fluorescence, e.g. substituted flavones, coumarins and umbelliferones; caffeic acid, chlorogenic acid, scopoletine, coumestrol, etc.

Many vitamins or their derivatives possess "blue" fluorescence (around 450mu) e.g. vitamin A, lumiflavin from riboflavin, thiochrome from thiamine, pyridoxine derivatives, folic acid, folinic acid, etc. The vitamins cyanocobalanin (vitamin B_{12}) and thiamine have been measured in seawater and it is known that many marine bacteria produce vitamins whilst certain green and blue-green algae (freshwater) contain appreciable amounts of vitamins (e.g. Chlorella pyrenoidosa, Anabaena cylindria etc.). In addition, seaweeds are rich in vitamins which are bound to be released in coastal waters either by excretion or decomposition of the seaweeds - bacteria decomposing the seaweeds will also contribute vitamins.

Many of the above blue fluorophors (which only represent some of those that could be present) are no doubt of limited stability and generally would not be in high concentrations, however under certain circumstances they might reach appreciable amounts (e.g. after a plankton bloom or in areas where seaweed is abundant). In addition, the intensity of the fluorescence of some of these compounds is very high, e.g. umbelliferone (7-hydroxy coumarin) is one of the most intensely fluorescent compounds known.

(c) UV FLUOROPHORS

A number of aromatic polycyclic hydrocarbons fluoresce in the near ultraviolet region, and as mentioned above some of these have been extracted from seawater (see (b)).

The amino acids tryptophan and tyrosine fluoresce in the UV region (the fluorescence of proteins is due to these amino acids which are linked to the protein molecule). Tryptophan fluoresces maximally at 348mu when excited at 287mu while tyrosine fluoresces maximally at 303mu when excited at 275mu. The fluorescence of tryptophan is greatest at about pH 11 when it is about 100 times greater than that of a comparable concentration of tyrosine. Both of these amino acids are commonly found in fresh and seawater.

In addition, these amino acids give rise to a large number of metabolites that fluoresce in the UV region, (e.g. indoles which are the most highly fluorescent of the biologically occuring compounds). Tyrosine gives rise to tyramine (same fl. as tyrosine), the phenolic acids p-hydroxyphenylpyruvic acid and homogentisic acid (ex. 290mu and fluorescence 340-345mu), 3,3-dihydroxyphenylalanine (dopa- the parent substance of melanin on one hand and of the physiologically important epinephrines on the other) dopa fluoresces at 325mu when excited at 285mu, etc. Tryptophan gives rise to tryptamine and indole acetic acid, which are both widely distributed in the plant kingdom. Indole acetic acid has been found in algal extracts in high concentrations, (Augier, 1965).
The fluorescent characteristics of these compounds are the same as those of tryptophan.

Tryptophan is also hydroxylated to 5-hydroxytryptophan which is decarboxylated to 5-hydroxytryptamine (serotonin). This latter substance is also widely distributed in nature, both in plants and animals, in neutral or slighly acid solutions it fluoresces at 330mu when excited at 295mu, a new peak is obtained at 550mu with addition of HCI (the peak at 330mu is suppressed in 3N HCl).

The aromatic amino acids are all fluorescent but they vary widely in their ability to yield measurable fluorescence in solution, Teale and Weber have reported their spectral characteristics in water (1956). Many of these acids fluoresce in the UV region and can reach relatively high concentrations in both fresh and seawater (but are very rare in bog water according to Swain, Blumentals & Miller (1959)).

Purines and many of their derivatives fluorescence in the UV, e.g. guanine and its derivatives fluoresce around 360 to 390 when excited at 272-280mu (the intensity is about 1/10th of that of a comparable concentration of tryptophan, as well, pH and the presence of other ions are critical for purine fluorescence).

With the exception of thymine the pyrimidine compounds do not seem to have any fluorescence. Thymine fluoresces maximally at pH 11 with excitation maximum at 290mu and fluorescence maximum at 380mu (it is less than 1/10th the intensity of trytophan fluorescence at same concentration).

Finally, UV fluorescence is characteristic of phenols and catechols (excitation maximum at 285mu, fl. maximum at 325mu) which are widely distributed in nature.

(d) "RED" FLUOROPHORS

In vivo chlorophylls and chlorophylls dissolved in a solvent such as acetone fluoresce in the red region of the spectrum. Degradation products of chlorophyll such as phaeophytin and other pigments such as those of phycobilin and various porphyrins also fluoresce in this spectral region.

In seawater chlorophylls *a* and *c* and phaeophytin will be the major pigments. Detrital chlorophyll is best characterised by phaeophytin which is readily formed by passing cells through the gut of zooplankton or by the action of bacteria or acid. The maximm peaks for *in vivo* chlorophylls are: excitation 436, fluorescence 685mu. Chlorophyll *a* dissolved in 90% acetone has the peaks: excitation 430mu, fluorescence 670mu; chlorophyll *c* has the peaks: excitation? , fluorescence about 632mu. Phaeophytin apparently has similar spectral characteristics to chlorophyll *a*. The fluorescent intensity of these pigments *a*:*c*: phaeophytin are in the ratios 100:88:42 respectively. The fluorescence of *in vivo* chlorophyll is considerably less efficient than that of dissolved chlorophylls, yielding only about 1/10th as much fluorescence per unit weight as the same amount in solution.

In fresh water the concentration of other pigments, e.g. chlorophyll *b*, will become more significant.

(e) OTHER FLUOROPHORS

A "background" fluorescence in the spectral area around 570mu appears to be common in turbid coastal and estuarine waters (see part III). While $UO_2(CO_3)_3^{4-}$, which makes uranium rather unreactive in the marine environment, has a bright yellow fluorescence.

III. Light-absorbing Substances and fluorophors as indicators

It is now well established that optical measurements can make valuable contributions to the solution of many oceanographic problems; Jerlov (1963) has given an excellent review (with references) on the subject. Among such measurements are those concerned with the light-absorbing and fluorescent substances.

(a) WATER MASSES

Kalle (1937 and onwards) has investigated the relationships between yellow substances (YS) and blue-fluorescing substances and properties of seawater such as colour and salinity and has studied the formation of these substances and their optical and chemical properties. He (1949) measured the salinity and fluorescence of water samples collected from the Bothnian and Finnish Gulfs and estimated the concentration of YS in these samples (by extraction and optical measurements). From the relationships that he found between salinity, YS and fluorescence he was able to characterise four water types (continental water, typical Baltic water, rain-water and Kattegat water) and to explain the broad features of their mixing.

From the results of his light attenuation measurements in the Kattegat area Joseph (1955) showed that the content of yellow substances and the salinity may equally be used as an index to characterise the water masses. He pointed out that this should also be possible for oceanic areas where differences in YS occur, particularly for the oceanic "upwelling" areas and the estuaries of great rivers. Thus, two water masses of different colour (or content of YS) but not in the content of suspended substances, can be distinguished because one water mass will have a greater attenuation in the blue region of the spectrum than the other water mass. In the Kattegat area the low-salinity, high-coloured Baltic water mixes with the high-salinity North Sea water which is poor in yellow substances.

Jerlov (1955) showed that the absorption coefficient of YS at 380mu may be expressed as a linear function of the attenuation coefficients observed *in situ* at 380mu and at 655mu. This function was valid for both Baltic and Bermuda waters (Ivanoff, Jerlov and Waterman, 1961). Jerlov studied the distribution of YS in Baltic waters which enabled him to localise the fresh-water tongues near the river mouths and their shift with the currents induced by wind and weather. By comparing its distribution with that of salinity he found, as Joseph had, that the linear relationship between concentration of YS and salinity could be used to characterise water masses and study mixing processes.

Besides taking into account the attenuation due to the water and that due to scattering by suspended particles, it is necessary in such measurements to allow for the selective absorption by particles. Jerlov found that in waters off Bermuda particle absorption was greater than the absorption by yellow matter. Yentsch and Ryther (1959) and Yentsch (1960) also stress the importance of absorption in the blue by the photosynthetic pigments of phytoplankton. Yentsch (1962) measured the visible light absorption by particulate matter in the ocean and found that absorption in the blue was 3 to 5 times greater (up to 20 times in deep water) than absorption in the red. They also showed that a large fraction of the YS taken from Woods Hole waters is particulate or absorbed on particles.

Organic matter is known to be adsorbed on sand particles (Fox, Isaacs and Corcoran (1952); Jones (1960); Chave (1965)) and in some areas there are high amounts of suspended mineral particles at certain times of the year, e.g. English Channel (Armstrong 1958), while floating sand on lake and sea surfaces appears to be a common phenomenon. Meadows and Anderson (1966) found diatoms, bacteria, blue green algae and other micro-organisms and organic matter attached to marine and fresh water sandgrains.

Jerlov took the average particle absorption characteristics into account. To do this he multiplied the attenuation in the red, C_r, by a factor K which was determined from the experimental results. To obtain a measure of the absorption due to yellow substance he subtracted KC_r from the corresponding attenuation in the blue C_b. Typical values found for K ranged from about 1.6 to 2.0 (N.B. C_r and C_b are the attenuation values corrected for the effects of the water itself).

Malmberg (1964), using Jerlov's method, again showed the usefulness of YS determinations for characterising water masses and describing mixing processes. He worked in the Skagerrak.

Joseph (1949) measured the UV absorption down to about 360mu, Jerlov to about 310mu (1950). A number of workers have extended absorbance studies into the far ultraviolet:
Sournia (1965) gives a brief historical review of the absorption measurements done in the UV; apparently Tsukamoto was one of the first to carry out such measurements - in 1927 he measured the absorption of seawater at Roscoff in the region 234 to 212mu.

Armstrong and Boalch (1961) measured the UV absorption (200-400mu) of various samples of seawater and seawater culture solutions in which algae had been grown. They found marked differences between seawaters from different areas, the highest absorption (excluding that shown by algal extracts) was shown by coastal waters, especially in areas of plankton "blooms". The absorption at 220mu and 250mu by water at a station in the English Channel was recorded over a 17 month period. There were small seasonal variations, and variations that coincided with changes in salinity and silicate which Armstrong and Boalch believed to be due to

changes in the water mass at the station. They discussed what substances probably cause the major part of the absorption.

Sournia (1965) studied the UV absorption of coastal waters of Nossi-Bé (Madagascar). Three stations were studied, two at the mouth of the Bay of Ambanoro and one far inside the Bay and near mangroves. He found that the salinity was inversely related to the absorption at 265mu and that fluctuations in the absorption at each station followed a characteristic pattern that could be related to local and seasonal changes and, for the landward station, the influence of the tide. He concluded that the measurement of UV absorption is a simple and rapid way to gain information useful for characterising water masses and for estimating the relative amounts of organic substances present in the different areas.

Ogura (1965) measured the UV absorbance of estuarine and off-shore waters in the western North Pacific. He found higher absorbance coincided with the decreases in chlorinity and transparency, and increase in COD, indicating mixing with land drainage. He considered that absorbance $_{230}$/absorb.$_{220}$ would be useful for indicating the degree of pollution by land drainage.

As mentioned above Kalle used fluorescence as well as light absorption to characterise water masses and trace the outflow of river water into the sea. He found that the fluorescence and salinity were linearly related. Among the areas he studied were the Baltic (1949), the North Sea (1953,1959), the Bight of Heligoland (1956), the Irmiger Sea (1957) and (in collaboration with Postma) the Elbe River (1955).
Kalle (1963) has listed some comparative values of fluorescence for various natural waters, they range from about 0.4 for the Sargasso Sea up to 100 for water from Moorbach, Niedersachsen in Germany (the values are in "mFl", a unite introduced by Kalle: 0.1mg of quinine bisulphate per litre of N/100 sulphuric acid yields 73mFl - Pulfrich photometer of Zeiss, 0.8ml cuvette (Kalle 1963)).

Postma (1954) measured the fluorescence in the Gulf of Paria in an attempt to distinguish between brackish water mixtures from different estuaries (the area nearby the gulf has large forests and extensive marshes). He found only small differences in fluorescence intensity for the various tributaries of the Orinoco system but relatively marked differences between the values for the dry as compared with those for the rainy season, which were lower. Postma found linear salinity-fluorescence relationships related to the season. During the rainy season the high discharge from the rivers into the sea decreased the concentration of the fluorescent substances, which Postma assumed were added to the river water and brackish estuarine water at about the same rate the year round. Hence the fluorescence-salinity

relationships provided a good index of the influence of the rainy season. The higher fluorescence during the dry season might however be due to a higher rate of production of the fluorescent substances since the concentration of suspended matter was higher in the Gulf then. It is possible also that an excess of trace elements, washed in from the soil during the rainy period might have a quenching effect on the fluorescence.

Duursma (1960) measured the fluorescence of the waters of the Netherlands Wadden Sea and surface waters near the "Texel" lightvessel in the North Sea, 12 miles off the Netherlands coast. He found that the fluorescence and salinity for the Wadden Sea were linearly related. The water near the LV Texel which Duursma considered as fairly representative of seawater well outside the influence of the coast and the fresh water inlets, was sampled fortnightly for a year. The samples were analysed to study the annual variations in concentrations of dissolved organic substances in relation to environmental conditions. Duursma found no clear relation between the fluorescence and the other data (salinity, which remained fairly constant; concentration of dissolved organic C, N, P; chlorophyll; oxidisable matter; PO_4; particulate matter and ignition loss).

Otto (1966) has measured the "blue" fluorescence in the Southern Bight of the North Sea during February-March, 1966 and found linear correlations with salinity. He also made light attenuation measurements and found that the difference in the attenuations for blue and red light (relative to the clearest water sampled) was linearly correlated with salinity. These correlations were different for the water masses in the west and east of the area sampled.

Fluorescent techniques involving the introduction of dyes such as Rhodamine B have been used for studying dilution rates, mixing, etc. of influent waters but it is not intended to discuss this work here (see Pritchard (1960), Moon et al (1957), Carritt (1963)). It is of interest however that appreciable background fluorescence is sometimes recorded in these studies, especially in turbid estuarine and coastal waters. Rhodamine B shows maximum absorption at 550mu and maximum fluorescence at 575mu (the 546mu Hg line which strongly excites the fluorescence is generally used). This spectral area coincides with that characterising Shapiro's organic acids which suggests that the background fluorescence might be related to land drainage.

(b) THE BIOCHEMICAL CYCLE

As Kalle (1949) points out: more detailed knowledge of the relationships between fluorescence, concentration of light absorbing substances and other properties of seawater will help to - clarify the still obscure relationships between the various "humus substances" (especially as regards their behaviour under normal conditions in nature) as far as origin, change and decomposition of these compounds are concerned - and help in an understanding of the fundamental chemical - biological events in the sea which are particularly relevant and important to the productivity problems of the sea.

The potential value of optical measurements in biological oceanography is illustrated by the three objectives recommended by "the in situ light measurements working group of the Committee on Oceanography of the National Academy of Sciences - National Research Council" (1965), viz.
(1) determination of the amount of light available to phytoplankton for photosynthesis in seawater, (2) the use of light parameters to determine the ratio of living photosynthetic phytoplankton to nonliving particulate matter in situ, and (3) the use of in situ fluorescence determinations for the study of biochemical cycling of organic matter - fluorescence measurements are valuable in studies of production and decay of organic matter and may ultimately provide a simple means of determining the concentration of non-living organic matter. Information is needed as to the characteristics of, and the effects of physical and chemical factors on fluorescence excitation and emission spectra.

Fluorophors and yellow substances (YS) from plankton

As discussed previously, the "parent-substances" of Kalle's YS and blue fluorophors are carbohydrates. Provasoli (1963) has reviewed the work on excretion of carbohydrates from fresh water and marine algal cultures and points out that brown and red seaweeds produce large quantities of mucilaginous polysaccharides (e.g. alginic acid, agar, etc.).
It seems generally agreed that most of the "carbohydrate" is released from dead or dying cells (Duursma (1960), Provasoli (1963), Marker (1965)). Marker found increased production of carbohydrate when the environmental conditions became unfavourable (salinity changes, low light intensity, nitrogen deficiency).

Phytoplankton then, during and after their late stages in growth (and apparently during their early stage of growth also) release large quantities of carbohydrates. Lewis & Rakestraw (1955) found a direct correlation between CHO concentration and the occurrence of suspended organic matter in coastal lagoons.

Walsh (1965) studied dissolved carbohydrate in Cape Cod waters (coastal and estuarine) and concluded that phytoplankton plays a major role in the regulation of DCHO concentration in natural waters (and presumably therefore regulate the concentrations of CHO derived YS and fluorophors).

Other light-absorbing substances (whether identical with, related to or distinct from Kalle's YS) are known to be derived from plankton: Jerlov (1951) found that Red-Sea water contains a dissolved yellow or brown component presumably derived from the decomposition products of certain algae (Trichodesmium erythraeum). Sournia (1965) found high UV absorption in water containing a proliferation of Cyanophyte Trichodesmium near the mouth of the Bay of Ambanoro.

As mentioned in Part III(a), Armstrong and Boalch (1961) measured the UV absorption spectra (200-400mu range) of various seawater samples and seawater culture solutions in which algae had been grown. The highest absorption was shown by water from the Firth of Clyde collected during April at the time of a heavy bloom of the diatom Skeletonema costatum. The volatile UV-absorbing substance (from English-Channel water) showed marked seasonal fluctuations in concentration, and had an absorption spectra similar to that of distillates from plant cultures (Ectocarpus, Phaeodactylum, etc.). Armstrong and Boalch believed that this substance was derived from the phytoplankton.

Yentsch (1962) and Yentsch and Reichert (1962) found an inverse relationship between the disappearance of chloroplastic pigments in macerated algae and the production of yellow compounds that absorbed UV light. They have suggested that these yellow compounds are derived from the protein carrier originally coupled to the chlorophyll chromophore.

Apart from the dissolved fluorophors derived from plankton it should be mentioned that many small organisms are brilliantly luminescent, e.g. many dinoflagellates (Clarke and Kelly, 1965).

In the open ocean areas where precipitation is not excessive YS production will be entirely due to the plankton. The concentration in such areas can be very high, e.g. in the upwelling regions west of Africa and South America (which practically lack freshwater supply by drainage and precipitation) Jerlov (1951) found abundant YS.

Riley (e.g. 1956) has treated transparency in part as a biological variable. He found that a satisfactory equation for use in the open sea is: $k = 0.04 + 0.0088C + 0.054C^{2/3}$ where k is the attenuation coefficient of visible light and C is ug of chlorophyll per litre. Jerlov (1957) has indicated the usefulness of the transparency meter to trace accumulations of plankton or detritus in the upper strata in the ocean and to determine the amount of YS which serves as an indicator of the organic production.

Kalle (1959) examined sections of the North Sea during the summer of 1950. In the areas sampled strong thermoclines form during the spring and summer and the residue from the phytoplankton bloom, which occurs during spring, collects along these thermoclines. Thus, Kalle found maximum chlorophyll content at 25 and 45m. The distribution of "blue" fluorescence of bacterial origin, mentioned on page 7, was closely associated with the distribution of the chlorophyll, to which it was reciprocal. The distribution of "free" fluorescence was similar but more indistinct.

In coastal and estuarine areas YS from land sources and precipitation might dominate over that derived from the plankton (e.g. in the Baltic area where production is not very high but drainage from the rivers, which are rich in YS, is high). However, in other areas the plankton may still contribute the higher proportion, or even all, the YS.

Duursma (1960) concluded that the dissolved organic substances and the associated fluorescence at LV Texel derived from the breakdown of the plankton growing in the area, i.e. that contributions from the mainland were negligible. This is supported by the fact that his data show the C/N ratios at various times of the year to correspond to the ratios of the plankton groups dominant at the corresponding periods. (e.g. the C/N ratio during August-November was around 8 and Peridinales (C/N 8.5) were the dominant plankton group; from November to end of May the ratio was low, ranging from 4 to 6, during this period copepods (C/N 4-5) and diatoms (C/N 6) were dominant. High ratios in June (C/N 11-33) might be due to higher temperatures and thus rapid breakdown of the organic nitrogen. Although Fraga (1966) found that high ratios indicated dissolved organic matter of recent formation.)
It is interesting to note also that Duursma's data seem to indicate a linear relationship between the ratio N/C and the fluorescence, which increases with the ratio.

The fluorescence measured by Ivanoff (1962) in the Tyrrhenean Sea and waters to the south-west of Corsica seems to be directly related to the plankton. Ivanoff found that the fluorescence increased from the surface to a depth of 100-200m then remained fairly constant down to 2000 or 3000m. At shallow depths (less than 75m) a correlation was found between the fluorescence and the light scattering coefficient of the water. These results can be interpreted as follows: YS and fluorophors are formed in the productive euphotic zone at a rate depending on the concentration of the section. Active growth occures in the upper layers and there is probably a balance between release and utilisation of the organics such as CHOs. This balance will be related to the number of plankton. The less active and dead plankton tend to sink to deeper layers where no such balance exists and where breakdown products of the CHOs and other organics will accumulate (according to Wattenberg (1937) disintegration of carbohydrates takes place between 200-600m in the ocean). The loss of correlation between particles and fluorescence is partly due also to the fact that the particles lose their discreteness, either breaking up into smaller particles or forming flocs. Apparently, at greater depths an equilibrium is established between supply and loss of the fluorophors.

With the information and techniques available at present it is difficult to distinguish between the fluorophors and YS of plankton origin and those derived from land sources. Yet if absorption and fluorescence measurements are to be truly useful in biochemical and characterising studies of water masses in coastal and estuarine areas, such a distinction is necessary. Parallel measurements of the chloroplastic pigments would be useful in this regard.

Chloroplastic pigments

The light-absorbing characteristics of the chloroplastic pigments have been the basis of their determination for many years. The pigments are extracted into an organic solvent and the absorption values determined at specific wavelengths, these values are then used in empirically derived formulae. More recently, fluorometric determination of the pigments has been made on both extracted samples and in situ while absorption measurements have been extended to in situ detection and estimation of the pigments.

Kalle (1951) measured the chlorophyll content of seawater by utilising the pigment's intensive red fluorescence. The chlorophyll was extracted from the particulate matter of seawater by means of acetone. This method is only now coming into wider use. The fluorescence of chlorophylls and breakdown products extracted into aqueous acetone has now been measured by: Yentsch and Menzel (1963), Lorenzen (1965), Holm-Hansen et al (1965), Yentsch and Lee (1966) and no doubt others. In vivo chlorophyll has been measured by a continuous flow method in seawater (Lorenzen, 1966).

Tyler (1964) has used spectroradiometric measurements to detect and estimate chlorophyll and other pigments in situ.

Metal chelators

There is evidence to suggest that some of the light-absorbing and fluorescent substances of natural water have a growth-stimulating action on certain groups of organisms (Shapiro (1957), Provasoli (1963)). While some (e.g. amino acid complexes) appear to be utilesd for photosynthesis, in preference to the inorganic forms of CO_2 (Smith et al 1960).

Shapiro (1957) found that this yellow acids had a stimulatory effect on the growth of certain algae (perhaps significantly, just those algae stimulated by soil extracts). He thought that this might be due to the solubilising ability shown by the acids toward iron (as many workers have pointed out, e.g. de Kock 1955 (cited by Shapiro), plants grow very much better when they are supplied with a mobile form of iron such as a soluble complex or chelate).

Provasoli (1963) has discussed the significance of metal chelators in the aquatic environment and reviewed some of the work done on this subject: see also Johnston (1964) and Duursma (1965). According to Johnston seawater quality (nutritive) is closely related to the chelation of trace metals, mainly iron, in fact the supply of chelating substances is frequently the most crucial aspect of phytoplankton nutrition in seawater.

Many organic substances isolated from natural waters (amino acids, peptides and proteins) form complex molecules with numerous metallic ions. Thus, in view of the frequent discrepancy between the high amounts of "trace" metals (e.g. iron) dissolved in natural waters and their solubility products (Laevestu and Thompson, 1958; Jones (1960)) it seems likely that complexes are formed. However, Duursma (1966) investigated the solubility of certain metals (Fe, Zn, Ni, Co and Cu) in seawater at different pH values but found no conclusive evidence of the chelating influence of natural organic compounds in seawater. He noted that higher concentration of iron might be due to $FeOH^{++}$ and $FeCl^{++}$ ions. Kent and Hooper (1966) have found that alkyl benzene sulfonates (common components of detergents) prevent the formation of iron chelates or complexes with amino acids at detergent concentrations greater than 0.3mg/l. (the amino acids appear to chelate with the detergent). Thus, in rivers polluted by sewage, domestic wastes, etc. the iron binding ability of amino acids are impaired or lost. This might also apply to other chelating substances besides amino acids.

The author of this review has found that soluble iron suppresses the "blue" fluorescence of various samples of natural water (pond and river water) which could indicate chelation. Some experiments of D. Eisma (personal communication) seem to indicate that easily chelated "blue"-fluorescent substances are only present in freshwater, while other "blue"-fluorescent substances present in both fresh and seawater can be chelated after attack with strong acid. It would be interesting to see what effect other metals have on this fluorescence and also what effect the trace metals have on the UV-fluorescence of natural waters.

Davis and Deller (1966) have demonstrated the strong iron chelating ability of dihydroxy acetone, and sugars with the dihydroxy acetone structure (substances that have been isolated from natural waters). This is particularly interesting since Kalle (1963) has shown that a "blue" fluorophor is formed from dihydroxy acetone. The DHA apparently undergoes an intramolecular change to form the volatile methyl glyoxal which is the basic substance involved in the formation of the carbohydratehumic acids and the associated blue fluorophors.

Methyl glyoxal appears to inhibit cell growth by arresting protein synthesis - iron might minimise this effect either by direct chelation or preventing breakdown to methyl glyoxal. On the other hand Fe might have a catalytic effect and accelerate the breakdown of methyl glyoxal once it is formed.

Surface activity

As mentioned above appreciable amounts of light-absorbing substances adsorb onto particles. Chave (1965) found that organic matter adsorbed onto mineral particles prevented the free interaction between seawater and the particles and hence the particles were kept in suspension. Such organic-coated suspensions might support bacterial flora as suggested by Armstrong and Francis (1958) who found appreciable quantities (maximum in winter) of finely divided claylike material in seawater from the English Channel. Another interesting feature of plankton substances is their stabilising effect on foam (Abe and Watanabe 1965).

(c) OPTICAL RATIOS AND INTERRELATIONSHIPS

A number of authors have pointed out the usefulness of optical ratios to characterise water masses or to indicate the probable origin of the organic substances in a particular area. Such ratios also provide useful information on the relative concentrations of different chloroplast pigments and on the various phases of biological activity. The ratios may be of absorption at different wavelengths, of absorption and fluorescence or fluorescence of different substances or fluorescence at different wavelengths.

Absorption

Kalle (1961) used the ratio K_{420}/K_{665} (where K is the absorption coefficient) to characterise his different "Gelbstoff" solutions. The melanoidines (glucose-melanoidin and dihydroaceton-melanoidin) had the highest ratios and the phenol-humic acids (pyrogallol-humic acid and hydrochinon-humic acid) the lowest. The ratios of the natural "Gelbstoff" solutions were between these two extremes, the seawater samples (Baltic Sea, North Sea, North Atlantic) having higher ratios than the fresh water samples (Lower Elbe, bog water). Kalle therefore concluded that seawater contains chiefly the yellow melanoidines and fresh water the brown phenol-humic acids.

Gorham (1960) compared the absorption curves of acetone extracts of surface muds from five English lakes. In the most infertile lake the bulk of the organic matter was derived from the lake's drainage basin and so the absorption curve closely resembled those of aerobic woodland mull and morhumus layers (characterised by a strong absorption at 350mu) while in the fertile lakes the large phytoplankton crops provided most of the organic matter and the absorption peaks of

chlorophyll derivatives (especially phaeophytin a) dominated the spectrum. Absorption curves were also obtained for living and dead phytoplankton and for detritus. The ratio of absorption at 410mu to absorption at 350mu was characteristic for the source of the organic matter e.g.
plankton (live) 2.9, plankton (dead) 2.3, detritus 2.0-2.1, very fertile lake 1.8, moderately fertile 1.0-1.4, and infertile lake 0.7. Unfortunately, neither Gorham nor Kalle extended their measurements into the far UV.

Ogura (1965) after investigating the UV absorbance of bay water and off-shore waters in the western North Pacific concluded that the ratio of the absorption at 230mu to absorption at 220mu would be useful for indicating the degree of pollution by land drainage.

As pointed out by Yentsch (1962) the difference in spectral absorption characteristics of the various chloroplastic pigments can be used to distinguish between healthy plankton and nutrient-deficient, moribund or decaying plankton. In healthy cultures the ratio K_{440}/K_{675} ranges from 1.3 to 1.5 whereas for seawater particulate matter the ratio ranges from 2.7 to 5.3 (the range is 3.5 to 5.0 for pigment deficient mutant leaves). The most stable components of the pigments system are carotenoids which absorb more strongly in the blue region (Yentsch and Ryther (1959), and Johnston (1955)) which explains the higher ratios for particulate matter. Distinction can also be made between the different plankton groups e.g. chlorophyll c and fucoxanthin absorptions characterise the diatom curve whereas chlorophyll b absorption characterises the spectral curve of the green flagellate while the marine bacterium Nitrosomonas has characteristic bands at 415mu, 521mu and 550mu (due to cytochrome C), etc. Herrera and Margalef (1961) found the ratio K_{430}/K_{665} useful in the study of the relationships between the concentrations of different pigments. The increase in the numbers of dinoflagellata was characterised by increase of relative amounts of chlorophylls b and c and of astacene carotenes and with increase of the ratio K_{430}/K_{665}. Earlier, Van Norman et al (1948) had found that certain algae had different relative absorptions at different wavelengths.

Smayda and Boleyn (1965) found that changes in ratios of pigment absorbance at various wavelengths (in mu: 352/474, 382/474, 404/474, 434/474, 414/352) accompanied changes in the rate of sinking of the plankton. This suggested that the rate is influenced by changes in adaptive physiological mechanisms of flotation beside the usual physical factors.

Absorption and fluorescence

The investigations of Kalle concerning the fluorescence and "Gelbstoff" contents of fresh and seawater (1949) indicated that water arising from marshy or peaty areas (e.g. Baltic or Elbe rivers) is characterised by a low fluorescence: absorption ratio relative to the ratio for ocean water. Kalle also characterised a special Baltic water type "0" by means of this ratio. Type "0" water was considered to be "old" in the sense that it had remained isolated in the Baltic basin, free from any appreciable mixing processes, over several years. This water had a ratio intermediate between the other two. The relative ratios are: ocean water: "0": bog water 8:2:1 approximately.

Kalle assumed the different ratios to be due to the different stabilities of the fluorescent and "yellow" substances (i.e. to differences in their chemical and biochemical behaviour).

In addition, Kalle (1963) has shown that the Fl/Absorp. ratio associated with the carbohydrate humic acids depends on the chemical system involved in the formation of the blue-fluorescent substances and the melanoidines. For an alkaline system of carbohydrate breakdown (into the fl. substance and "Gelbstoff") the ratio is fairly constant. For a system containing certain N compounds (such as amino acids) the ratio is higher but varies somewhat according to the amino acid/carbohydrate system. The relative values of the ratios for an alkaline system with ribose versus an alkaline system without N compounds is approximately 4:1. The ratio for the ribose system is 10 compared with 12.7 for the ocean water, although it is not certain whether the units used by Kalle are the same, and therefore these ratios cannot be strictly compared.

Carlucci and Williams (1965) concentrated bacteria from seawater by bubble scavenging. They found that increase in fluorescence (wavelengths larger than 415mu only recorded) and absorption at 270mu accompanied the concentration of the bacteria. They thought that this might indicate lysis of the cells in the foam fractions after various bubbling times (up to 4 hours) with concurrent release of organic material into the seawater medium. The ratio of fluorescence/absorp.$_{270}$ appeared to be higher for detritus than for fresh cells.

Fluorescence

Yentsch and Menzel (1963) and Lorenzen (1965) estimated the relative amounts of chlorophyll and phaeophytin present in pigment extracts from the "acid facor" (ratio of the initial red fluorescence to fluorescence of acidified extract - dilute acid removes the Mg from chlorophyll to form phaeophytin). Low ratios (1.35 or less)

indicate a high percentage of degraded chlorophyll, indicating bacterial decomposition or heavy grazing by zooplankton (the amount of phaeophytin in laboratory grown cultures is very small, but phaeophytin is readily formed by passing chlorophyll through the gut of zooplankton). A ratio around 2.4 indicates pure chlorophyll a. Ratios for other pigments such as chlorophyll c (6.1) have been obtained. These ratios should give much useful information on relative pigment concentration and provide a quick method of estimating the extent of zooplankton grazing.

A comparison of the fluorescence in different spectral regions should prove very useful for characterising water masses in more detail.

IV. Discussion

The spectral features of a water mass can be used not only to characterise its structure and hence distinguish it from other water masses but also to indicate the biological and chemical events that have occured within the water mass. Absorption and fluorescence measurements may therefore be conveniently divided into (1) those concerned with characterising and tracing water masses and (2) those concerned with the biochemical cycle.

(1)

Absorption and fluorescence measurements related to (1) have a number of inherent limitations. To estimate the absorption due to dissolved substances, attenuation by particles and the water itself must be taken into account. Also, it is doubtful whether "yellow substance", even though relatively stable, can be considered a conservative property. These difficulties have been discussed by Jerlov (1955) and Malmberg (1964), and recently Sournia (1965) has reviewed the attenuation contributions of the various components of seawater. As yet, there is not clear distinction between the fluorophors derived from marine or freshwater plankton or those derived from terrestrial sources. This introduces difficulties in interpretation when admixtures of fresh and seawater are involved, especially when no information on the biological activity in the area is available.
In addition, the problem of the non-conservative character of fluorescence as a property of a water mass has to be considered.

Despite these and other difficulties absorption and fluorescence measurements have already proved useful, especially in distinguishing between water masses whose other properties are very similar (e.g. temperature, salinity or particle content). However, the value of these methods, especially as regards fluorescence, have hardly been exploited yet. A major obstacle is the lack of information concerning the nature of the absorbing and fluorescing substances. The spectral characteristics of the absorbing substances are now better known (measurements having been extended to the far ultraviolet region) but those of the fluorophors (excluding chloroplastic pigments) remain vague, being generally defined as "blue" or "blue-green" or "green-yellow". The fluorescence measurements can hardly be made specific until the major spectral peaks are known. There are indications (from the results of Shapiro and some preliminary experiments by the present author) that stable fluorophors exist that originate from land drainage and therefore are not found in seawater unless it contains admixtures from the land. Such fluorophors would probably be more suitable as "tracers" of freshwater inflows to the sea than the blue fluorophors of Kalle which are universally distributed and have the same spectral range as many other biological substances.

To increase the usefulness of fluorescence measurements it is suggested that the fluorescence characteristics of fresh water, seawater and plankton extracts be investigated: What are the major fluorescence peaks (in the range 300-800mu say) of fresh samples and what modifications occur with time? What effects do pH and metal ions have on the fluorescence? (preliminary experiments have shown that ferric and ferrous iron quench the fluorescence of fresh water (in spectral region defined by Corning filter 9788, blue-green) and that the fluorescence intensity varies with the pH). Once the most useful spectral regions have been defined it should be possible to choose the appropriate region(s) for a particular investigation. This means that the most appropriate instrument set-up can be chosen (i.e. exciting and recording components that will give optimal results can be chosen: instrumental notes are given in the appendix). It should be possible to simultaneously record emissions in different spectral regions (e.g. 300-400mu, 550-600mu and 420-470mu, say), and hence characterise the water masses in greater detail. The measurements could be made in situ by a continuous flow method.

(2)

Udenfriend in his book "Fluorescence Assay in Biology and Medicine" (1964), makes it clear how extremely important fluorescence measurements have become in biochemistry. The usefulness of such measurements in analyses of the complicated chemical system presented by such fluids as blood suggest that they would be particularly suited to analyses of seawater. However, such measurements are not yet widely used in oceanography.

Fluorescence techniques have been found very useful in the estimation of various chloroplastic pigments, especially since the measurements can be easily made in situ. There is every reason to believe they will be equally useful not only in direct analysis for important biochemicals but in investigations of growth, photosynthesis, reproduction, decay, the rates of overturn of various substances, etc..

e.gs.
Estimating biomass: Krey (1959) has pointed out that the albumin equivalent may serve as a good indicator of the active substance in live plankton and of the organic substance as well. He thinks that determination of the albumin equivalent

should be useful in routine analyses at sea for estimating the standing crop of plankton and its mineralization potential. The characteristic fluorescence of proteins is sufficiently sensitive and specific to make it practical for use in quantitative assay. In addition, proteins conjugate with certain dyes which fluoresce only on adsorption to the protein (e.g. 1-dimethyl-aminonaphthalene 5-sulfonyl chloride). Fluorescence techniques (using dyes or the natural fluorescence) are now used not only to determine concentrations (e.g. albumin in serum (Rees et al, 1957), protein in milk (Konev, 1961) but also for tracing proteins in the body, investigating their structure, breakdown, etc. It should be possible to adapt one of these fluorescence techniques for estimating the amount of albumin in seawater, the advantages of such a technique being its simplicity and high sensitivity. (the natural fluorescence of proteins is UV region).

Breakdown of seston: Kalle has already found a relationship between chlorophyll and a blue fluorophor which accompanies the chlorophyll when it is extracted from the seston with acetone. This blue fluorophor seems to be derived from bacterial breakdown of the seston.
Measurements of CHO concentration and the blue fluorescence of water samples and plankton extracts might provide useful information on breakdown processes.

Growth: Sutcliffe (1965) has suggested that a measurement of ribonucleic acid (RNA) concentration might permit a prediction of growth rate even when applied in gross analysis to mixed populations, such as plankton. RNA is fluorescent when excited at 280mu and it should be possible to develop a fluorescence technique for determining its concentration.

Photosynthesis: Duysens and Amesz (as reported by Udenfriend, 1964) were able to show, by means of fluorescence techniques, that pydrine nucleotide reduction accompanies the reduction of carbon dioxide during photosynthesis.

Zooplankton grazing: The results of fluorescence measurements of the chloroplastic pigments have shown that fluorescence is useful for detecting nutritionally deficient plankton, and estimating the extent of zooplankton grazing.

Trace metals: Hoelzl, Wallach and Steck (1963) have developed a fluorescence technique for the micro-determination of metals in biological materials (Al, Co, Cu, Ni, Zn and alkaline earth metals) - it might be possible to adapt this for estimation of trace metals in seawater. Sackett and Arrhenius (1959 have used a fluorescence technique to estimate Al in the dissolved and particulate matter of seawater.

These are only a few of the possibilities!

Of course, fluorescence methods have certain disadvantages, as most methods have. Duursma and Rommets (1961) have treated the problem of "self absorption" mathematically, and have derived relationships which enable the absolute fluorescence to be determined; the absolute fluorescence is proportional to the concentration of the fluorescent substance(s) (as shown by application to fluorescence determinations of natural waters and standard solutions). Udenfriend (1964) has discussed the problems relating to fluorescence analyses in general while Lorenzen (1965) has discussed those relevant to the determination of chloroplastic pigments. However, most of the problems can be overcome and are more than compensated for by the ease, quickness, and sensitivity of fluorescence measurements.

APPENDIX

Notes on Instrumentation:

Chloroplastic pigments

Acetone extracts

Holm-Hansen et al (1965) used an Aminco-Bowman recording spectrofluorometer to obtain the emission and excitation spectra of extracted chloroplastic pigments.

The concentration of pigments was calculated from the measurements with a Standard Turner fluorometer (G.K.Turner Associates, East Palo Alto) equipped with either:

1) a high-output lamp (110-853) and Corning filters CS-5-60 (excitation) and either CS-2-60 or CS-2-64 (light screening).

or

2) F4T4-B lamp, high sensitivity door, a Wratten 47B blue filter (for excitation light) and Wratten red 25 filter (to screen emitted light).

They used a Bausch and Lomb 505 recording spectrophotometer to determine the spectral transmission characteristics of the filters.

In vivo

Lorenzen (1966) used a Turner fluorometer model 111 equipped with a red sensitive photomultiplier (extends response to 750mu), blue fluorescence lamp F4T5 and Corning filters CS5-60 (primary) and CS2-64 (Secondary).

Udenfriend (1964) has reviewed the various types of instruments available for fluorescence measurements, and discussed the structure and scope of many of them. Fletcher (1963) has described an experimental model of a simple vertical-axis transmission-type filter-fluorometer.

The Turner fluorometer is a reliable instrument suitable for shipboard measurements. It is a filter fluorometer and can be adapted (by using appropriate light source, photomultiplier, cuvettes, etc.) for use in the UV, blue or red spectral regions. Various accessories are available to adapt for various types of measurements, e.g. micro, continuous flow, etc.

The standard model (110 or 111) is equipped with a lamp with peak emission around 360mu and an S-4 photomultiplier (RCA 931A) which responds down to about 650mu (need a special photomultiplier for longer wavelengths).

Some examples of suitable set-ups for different spectral regions are given below, although the choice of primary and secondary filters will depend on the spectral characteristics of the substance(s) being assayed.

UV region

The following equipment would be suitable for the fluorescence range 300-360mu.

Turner model 110 or 111 equipped with:

lamp 110-851: major emission wavelength at 254mu, with useful output at 297, 313, 405, 436 and 546mu - primarily used for far UV (254 and 297mu). A quartz non-fluorescent cuvette must be used with this lamp.

filters: primary - Corning 7-54 (254 to 420mu peak transmission) plus Turner 110-815, a special plastic filter for use with 7-54. The combination transmits 254mu but does not transmit wavelengths larger than 300mu

Secondary - Corning 7-54 + Wratten 34A (narrow pass with peak at 325mu)

Blue region

The standard Turner model with lamp and photomultiplier normally supplied. Equipped with the following filters:

primary - 7-60 (narrow pass, peak at 365mu)

secondary - Turner 2A (sharp cut, transmits below 415mu) and/or suitable Corning blue filter (e.g. for 400-500mu CS5-61 or CS5-57 would be suitable)

Yellow-green

The standard Turner model (as above) is suitable, equipped with the following:

lamp 110-851: this has useful output at 546 and 436mu. The best of these two wavelengths can be found empirically and an appropriate primary filter chosen (Rhodamine-B's fluorescence is highly excited by the 546 line - Corning 1-60 + Wratten 61 are used as a combination for the primary.)

secondary - for emission around 570mu, Corning filters 3-66 and 4-97 would be suitable.

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