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ON THE UNSAPONIFIABLE MATTER AND PHOSPHATIDE IN MARINE ALGAE FAT

By

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Introduction

The Chemical researches on marine algae are very few in comparison with those on land plants. Work in this field has been confined largely to the study of inorganic constituents, carbohydrates and allied substances, and only of late years have researches been made concerning other constituents.

However, on the fatty constituents^{(21) (27)} of certain species, especially on the unsaponifiable matter of fat we can find only a few works up to date.

Lately M. SUMI⁽²⁶⁾ separated sterol from *Enteromorpha* sp. (the green laver), *Hijikia fusiforme* (Harv.) OKAM. and *Digenea simplex* (Wulf.) Ag. to examine the distribution of ergosterol in them. According to his report the sterol obtained from these seaweeds was mostly sitosterol with a little mixture of ergosterol in each case.

The author has separated a kind of sterol from *Pelvetia Wrightii* (Harv.) YENDO and *Fucus evanescens* Ag. and named it pelvesterol.⁽²²⁾

Later HEILBRON, PHIPERS and WRIGHT^{(3) (6)} separated a kind of sterol from *Fucus vesiculosus* (the bladder wrack) and they thought the sterol to be peculiar to seaweeds and styled it fucosterol.

But judging from the properties of its derivatives and other points, this is quite identical with that styled pelvesterol by the author.

Afterwards the author separated the same sterol from *Laminaria ochotensis* MIYABE, *Hijikia fusiforme* (Harv.) OKAM. *Alaria crassifolia* KJELLM. and *Cystophyllum hakodatense* YENDO thus finding that its distribution is considerably broad. At the same time it was recognized that it has a property as a special physiological function, activating strikingly the action of lipase. On the other hand, HEILBRON and his collaborators prosecuted their study of the chemical structure of fucosterol along almost the same line as the author pursued from which an interesting relation was found between the activating function upon ferments and the constitutional formula of sterol.

The author has separated also pelvesterol-like substances that strongly activate the lipase more than pelvesterol and have cramp and narcotic actions similar to liver oil toxins.

Further, the author has newly separated a crystal that has an empirical formula $C_7H_{10}O_2$ and melts at $149^\circ C.$, various unsaturated hydrocarbons and a special phosphatide.

Moreover it is found that the brown substance of *Phaeophyceae* takes a combination form like that of a phosphatide and the brown pigment contained in it has the function to promote or restrain the growth of microbes.

In this treatise, the properties of these substances will be reported.

The author avails himself of this opportunity to acknowledge his sincere obligations to Prof. EIJI TAKAHASHI who directed and advised him while this work was being carried on.

Further, the author deems it his pleasant duty to offer his hearty thanks to Prof. YUKIO YAMADA who favoured him with invaluable instructions on the botanical side of seaweeds, Mr. TIYOITI KANDA, Rigakushi of the Muroran Institute of Algological Research, Faculty of Science of the Hokkaido Imperial University who afforded him much convenience in collecting seaweeds, Dr. MIZUHO SUMI of the Physico-Chemical Institute who gave him spectrograms and the sample of ergosterol, Mr. EIITI MAKINO, assistant professor, and Mr. KENITI DOI, Rigakushi, both of whom helped in taking the photographs of crystals and spectra, and to Mr. SADAHIKO YOSHIMURA, Nogakushi, who assisted in the microbe experiments.

Lastly, but not least, the author's profound gratitude ought to be expressed to the Tosyo-gū Tercentenary Commemoration Society which gave him a subsidy for the study of the theme of this treatise.

I. Pelvesterol

A. Preparation and purification of pelvesterol

1) Preparation of pelvesterol from *Pelvetia Wrightii* YENDO.

The algae used were collected in the summer of 1932 at the neighbourhood of Akkeshi Marine Laboratory of the Hokkaido Imperial University. After drying, the seaweeds were powdered finely, the residue extracted twice with petroleum ether (b.p. $40-50^\circ C.$). The crude fat thus obtained was deep green and when a large quantity of acetone was added to it, no precipitation occurred. After the saponification of the fat for 50 minutes with a 10%

alcoholic solution of caustic potash, the unsaponified matter was separated by the usual method. Putting the matter once more to the saponifying process, the unsaponifiable matter was completely separated. It was yellowish red and contained a large quantity of silk-fiber-shaped crystals. So by dissolving in a small quantity of hot strong alcohol, several re-crystallisations were carried out by which colourless needle-shaped crystals were obtained from the top solution. The base solution was deep dark red in colour and contained beside carotnoidal pigment a small quantity of needle-shaped crystals having low melting point. The melting points of the crystals obtained as above were as follows:

Crystals	M.p.		Crystals	M.p.
(a) 0.20 g.	119–120°	} Mixed together and recrystallised → A {	(1) 1.79 g.	122°
(b) 2.00 „	119–121°		(2) 1.40 „	121°
(c) 1.28 „	118–119.5°		(3) 0.60 „	119–120.5°
(d) 0.52 „	119–120°			
(e) 0.53 „	119–120°			
(f) 0.35 „	114–115°	} do → B {	(1) 0.88 „	120–121°
(g) 1.10 „	116–119°		(2) 0.17 „	119–120°

(1) are the crystals obtained from the top solution. (2) those obtained from the base solutions of (1), and (3) those obtained from the base solution of (2).

From the mixture of the base solution of A (3) and B (2) 0.23 g. of crystals of melting point 118–120° was obtained. The melting point of A (1) did not rise over 122° even though it was recrystallised. An acetate was prepared and the saponification and recrystallisation by the following method was carried out. The purified crystals thus obtained melted at 122°. 0.2055 g. of them was dissolved in chloroform to make up 5 c.c. and the rotatory power was estimated in a 1 dm. tube reading -1.60° , i.e. $[\alpha]_D = -39.0^\circ$. The solution did not give the reaction like that of ergosterol.

Acetate. The sterol was dissolved in 10 c.c. of acetic anhydride and heated on a water-bath for 45 minutes under a reflux condenser. After 3 hours it was poured into a large quantity of water and by shaking with ether the acetate was transferred into the latter. After the ether was removed, dissolved in absolute alcohol and recrystallised into colourless hexagonal plate-shaped crystals which melted at 118°. It was dissolved in chloroform to make up 5 c.c., its rotatory

power was estimated in a 1 dm. tube. The reading was -2.20° i.e. $[\alpha]D = -44.1^\circ$.

Propionate. The sterol was added to a little quantity of propionic acid anhydride and heated on a water-bath for 2 hours proceeding as before. After it was allowed to stand for 3 hours, the crystals were separated and by recrystallisation from absolute alcohol colourless hexagonal plate-shaped crystals were obtained. Their melting point was 104° .

Benzoate. The sterol was dissolved in a small quantity of pyridine, benzoyl chloride was dropped in it and kept at ordinary temperature for 3 hours. By adding water separated the glutinous matter which was recrystallised from absolute alcohol. The crystal was colourless square plate its melting point showed 114° .

Bromide. 1.2 g. of acetate were dissolved in 7 c.c. of ether and dropped in it 19 c.c. of 5% glacial acetic acid solution of bromine. Being allowed to stand on snow, white precipitate was separated. When the precipitate was washed with glacial acetic acid and cold absolute alcohol, white light powder was obtained. It was decomposed at 132° .

It was not capable of being recrystallised from absolute alcohol. When heated, it was decomposed into substances which had diverse lower melting points. Several attempts were made to obtain original acetate by reducing one of the decomposed substance (m.p. 132°) with zinc dust, but all in vain. Bromide has a very unstable character.

2) Preparation of pelvesterol from *Fucus evanescens* AG.

Pelvesterol from the sample which was gathered in spring (in May 1934 on the coast of Nemuro) and in autumn (in Oct., 1933 on the coast of Akkeshi), were separated and its nature was investigated by the same method as described above in *Pelvetia wrightii* YENDO in each case.

Unsaponifiable matter was fractionally crystallised from absolute alcohol.

Crude fats were used to the amount of 200 g. from spring seaweed and 50 g. from the autumn for the crystallisation. The results were as follows:

Spring weed $\left\{ \begin{array}{l} A_1 \text{ 6.5 g. (Yellow crystallised substance)} \\ A_2 \text{ 1.2 g. (Reddish yellow crystallised substance)} \\ A_3 \text{ 3.5 g. (Red substance, containing much low melting point substance which mixed with wax, alcohol etc., seemed wet in room temperature)} \end{array} \right.$

Total amount 11.2 g. (5.6% for crude fats)

Autumn weed $\left\{ \begin{array}{l} B_1 \text{ 2.23 g. (Yellow crystallised substance)} \\ B_2 \text{ 0.5 g. (Red. Same as } A_3) \end{array} \right.$

Total amount 2.73 g. (5.5% for crude fats)

A_1 and B_1 are the crystals obtained from the top solution.

A_2 and B_2 those obtained from the base solution of A_1 and B_1 .

A_3 those obtained from the base solution of A_2 .

The total amount of crystals separated from the unsaponifiable matter was almost the same in each case.

To crystals of A_1 , acetic acid anhydride was added and heated for 45 minutes. The colour of the solution was at first yellow and gradually became brownish. After cooling, the crystals separated were somewhat greenish. After 3 hours standing, steryl acetate was separated from absolute alcohol. Five grams of slight yellowish crude crystals were obtained which corresponded to 77%.

B_1 were treated the same as above. Saponified solution was at first yellowish and then gradually reddish brown. At the end of saponification they had no colour. After cooling, crystals were separated as above described. Yields were 1.50 g. which corresponded to 70%.

The above acetate crystals were recrystallised from absolute alcohol separately and both crystals were purified to hexagonal colourless plate showing m.p. 118°C. After being saponified for 30 minutes, free sterols were recrystallised from absolute alcohol which had m.p. of 122°. Above data are the same as for pelvesterol. 0.2427 g. of sterol dissolved in 5 c.c. of chloroform and measured optical rotation with 1 dm observation tube. It gave the reading of -1.930° , $[\alpha]_D = -39.8^\circ$.

Acetate. 0.2545 g. of the crystals (m.p. 118°) was dissolved in 5 c.c. of chloroform and optical rotation power was measured: $[\alpha] = -43.2^\circ$.

Propionate. It was treated the same as in *Pelvetia W.* The crystal with m.p. 103° was obtained.

Benzoate. m.p. 114°.

As above mentioned, a new sterol was separated by author from *Pelvetia Wrightii* YENDO and *Fucus evanescens* Ag.

This substance was colourless in the chloroform solution of trichloroantimony and did not turn red even when it was allowed to stand all day long. It was negative in the test of TORTELLI-JAFFE's reaction and did not show the reaction of ergosterol. When it was tested by LIEBERMANN-BURCHARD's reaction, the chloroform layer turned directly reddish brown and the acid layer turned red. In the test by Salkowski's reaction the chloroform layer turned violet and the acid layer red. The derivatives of the sterol were prepared and their properties were reported in an annual meeting of Sapporo Agriculture and Forestry Society in 1933. Later (1934) ⁽⁶⁾ HEILBRON and his collaborators reported in "Nature" on fucosterol.

In comparing the properties of two sterols, we find quite identical as shown in the following table.

TABLE I. Properties of new sterol and its derivatives.

	Sterol M.p. [α]D	Acetate M.p. [α]D	Propionate M.p.	Benzo- ate M.p.	Bromide of acetate M.p.
New Sterol - <i>Pelvetia W.</i>	122° - 39.0°	118° - 44.1°	104°	140°	132° (Decomposed)
- <i>Fucus e.</i>	122° - 39.8°	118° - 43.2°	103°	114°	-
Fucosterol (<i>Fucus v.</i>)	124° - 38.42°	118° 119° - 43.8°	105° - 106°	120°	133° (Decomposed)

Such sterol has never been reported. The author thought it to be peculiar to the seaweeds and styled it pelvesterol.

3) Preparation of Pelvesterol from *Laminaria ochotensis* MIYABE.

The seaweed that was used as material was commercial Hanaori Kombu (*Laminaria ochotensis* MIYABE). It was dried and finely powdered and treated in the same manner as the above mentioned. The crude fat gave a yellow colour and a particular smell. Eight grams of unsaponifiable matter were obtained by saponification of 150 g. of crude fat. It was dissolved in strong alcohol, decoloured with animal charcoal and crystallised by concentration.

$A_1=2.1$ g. (colourless crystals)

$A_2=1.2$ g. (pale yellow, containing wax alcohol of low melting point and seeming moist in ordinary temperature).

The crystals obtained by recrystallisation from A_1 melted at 122° . The acetate was prepared and saponified and sterol was recrystallised. Its melting-point was 122° as usual.

Its three derivatives prepared similarly as before showed melting-point as follows:

Acetate— 118° , propionate— 104° , benzoate— 114° , with crude pelvesterol, chloroform solution of trichlorantimony gave a pale red coloration, but no TORTELLI-JAFFE's reaction.

4) Preparation of Pelvesterol from *Hijikiya fusiforme* (Harv.) OKAM.

Hijikiya fusiforme (Harv.) OKAM. used as material was collected at the seashore of Kamogawa, Tiba. 12.5 kg. of the powdered sample were extracted with ether and 100 g. of crude fat were obtained. The fat was saponified with alcoholic sodium hydroxide solution by the ordinary method (22 g. of caustic soda was dissolved in 75 c.c. of water, 150 c.c. of alcohol was added, and the fat being put in the solution was heated for an hour). Then the alcohol was removed as much as possible, poured with stirring into water and the unsaponifiable matter was extracted with ether. The ethereal solution gave a beautiful red colour, and when the ether was removed there remained a yellowish red crystalline substance to the amount of about 21 g. It was saponified once more and the yields amounted to 20 g. By several recrystallisations from ethyl alcohol 9 g. of whitish crude crystals were obtained.

The acetate was prepared by treating the crystals in the same manner as above stated and purified from ethyl alcohol. From the top solution 5 g. of white crystals (melting point 117.5°) were obtained and saponified with 5% alcoholic alkali, and pure white pelvesterol was separated by recrystallisation from ethyl alcohol. Its properties were as follows:

m.p. 122° , $[\alpha]_D -39.6^\circ$ (0.1722 g. dissolved in 11 c.c. of chloroform. $\alpha_D = -0.62^\circ$).

The acetate of the pelvesterol obtained was prepared again. Its properties were as follows:

m.p. 118.50°, $[\alpha]_D -44.1^\circ$ (0.1817 g. dissolved in 5 c.c. of chloroform. $\alpha_D = -1.60^\circ$).

The propionate was prepared too. Its properties were as follows:

m.p. 105°–106°, $[\alpha] -43.1^\circ$ (0.1600 g. dissolved in 5 c.c. of chloroform. $\alpha_D = -1.38^\circ$).

The colour reactions of ergosterol (reactions to trichloroacetic acid, trichloroantimony, JAFFE's reagent and acetate of mercury) were absolutely negative.

Properties of pelvesterol separated from *Hijikia fusiforme* (Harv.) OKAM.

	M.p.	$[\alpha]_D$
Pelvesterol	122°	-39.5°
Pelvesteryl acetate	118.5°	-44.1°
Pelvesteryl propionate	105°–106°	-43.1°

5) Preparation and purification of pelvesterol from *Alaria crassifolia* KJELLM.

The seaweeds used as materials were collected July 1936 at the seashore near the Muroran Institute of Algological Research of the Hokkaido Imperial University. Soon after the collection, they were divided into a blade (A), and a sporophyll (a special blade on which reproductive organ is formed) (B). After dried under 70–80°C. it was powdered finely. Out of 40.5 kg. of A and 15.5 kg. of B obtained, 3 kg. were taken respectively and digested separately in 3.5 l. of 80–90% alcohol for 3–4 hours on a water-bath, and the infusions filtered severally. To each residue 2 l. of alcohol were added and digestions were carried out once more as before.

The filtrates were allowed to cool, the crystals of mannite and other impurities separated out were removed, on expulsion of the alcohol by concentration the filtrates were shaken up with small quantities of water and ether and the part soluble in ether was separated. After dehydration with anhydrous sulphate of soda the ether was removed and then phosphatide (below) was also removed by adding twice as much acetone to the remained solution.

The acetone solution was concentrated to expel the acetone and crude fat was separated. The fat was saponified with 10% alcoholic solution of KOH and the unsaponifiable matter was separated. The latter was dissolved in absolute alcohol, refrigerated with ice and

salt and the sterol crystallised out was separated by filtration. Yields: A. 9 g., B. 6 g.

Purification. The reddish-yellow crude pelvesterol was once more recrystallised from alcohol and acetylated with acetic anhydride,* the acetate was recrystallised from absolute alcohol. Pelvesterol was liberated by saponification. The yield was 6 g. for A and 3 g. for B. They were again acetylated and after recrystallisation from absolute alcohol, saponification and recrystallisation were carried out in the same manner as before and the following results were obtained:

- A. Pelvesterol 4.5 g., m.p. 122°
- B. Pelvesterol 1.5 g., m.p. 122°

The pelvesterol obtained above was once more purified by further acetylation and successive saponification. The yields and properties were as follows:

- A. Pelvesterol 3.7 g., m.p. 124°
- B. Pelvesterol 0.5 g., m.p. 124°

When A and B were once more recrystallised from absolute alcohol respectively they showed almost no change in their melting-point.

- A. Pelvesterol 3.2 g., m.p. 124° $[\alpha]_D - 41.6^\circ$
- B. Pelvesterol 0.2 g., m.p. 124° $[\alpha]_D - 41.3^\circ$

A part of each pelvesterol was taken and acetylated once more and there occurred changes in their properties as follows:

- A. Pelvesteryl acetate m.p. 119.5° $[\alpha]_D - 46.9^\circ$
- B. Pelvesteryl acetate m.p. 119.5° $[\alpha]_D - 46.5^\circ$

When the mother liquor of recrystallisation from acetic anhydride mentioned above was poured into a large quantity of water there was produced a yellowish granular substance. On filtration and through washing with water it was dried and a small quantity of a powder was obtained, which easily dissolved in alcohol and melted at 60–63°. (Its properties will be described below).

All the alcoholic base solutions of the recrystallisations of pelvesterol were added together and concentrated, and some crystals

* Acetic anhydride was added at the ratio of 10 c.c. to 1 g. of pelvesterol, and the solution was heated for 45 minutes on a water-bath, allowed to stand for 3 hours, poured into a large quantity of water, and ether was added to extract pelvesteryl acetate. On dehydration ether was removed.

were obtained. A part of them was twice recrystallised from alcohol and the crystals separated last melted at 122°.

In the course of the purification, the author's attention was attracted to the fact that the B had higher solubility than the A, but there was hardly any difference between them in their colour reactions.

6) Preparation and purification of pelvesterol from *Cystophyllum hakodatense* YENDO

The seaweeds used as materials were collected together with the above mentioned *Alaria crassifolia* KJELLM. They were dried and pulverised, and from 27 kg. of the powder 18 g. of crude pelvesterol were separated by the same procedure as applied to *Alaria crassifolia* KJELLM.

Purification. By the same method as used in the case of *Alaria crassifolia* KJELLM. pelvesteryl acetate was prepared and recrystallised from absolute alcohol one time. Its yield was 9 g. and its melting-point 118–119°. The acetate was saponified and pelvesterol was liberated. Its yield was 7.5 g. and its melting-point was 122°. The 5.5 g. of the pelvesterol were acetylated again, recrystallised from acetic anhydride one time, and the crystals were separated by filtration, washed with acetic anhydride and water, and recrystallised from absolute alcohol two times. Then the acetate was saponified and the liberated pelvesterol was recrystallised from absolute alcohol two times. Its yield was 3.3 g. and its melting-point was 123°. A part of the yield was recrystallised from acetone once more, but no change in its melting-point was seen. Then with a part of the crystals pelvesteryl acetate was prepared. Its properties were:

Pelvesterol m.p. 124° $[\alpha]_D - 41.5^\circ$

Pelvesteryl acetate m.p. 119.5° $[\alpha]_D - 46.6^\circ$

When the base solution of the recrystallisation from acetic anhydride was poured into a large quantity of water, there occurred a yellowish granular substance. It was separated by filtration and on being thoroughly washed was dried in a desiccator *in vacuo* over sulphuric acid. Its yield was 0.37 g. and its melting-point 61–63°. It dissolved very easily in alcohol and quite coincided in appearance with the substance obtained in the case of *Alaria crassifolia* KJELLM.

Both these substances were added and saponified (a gold coloration was given with alcoholic potassium solution), and the crystals

obtained were purified by repeating crystallisation from acetone. Yellowish crystals (m.p. 76–79°) were first produced and from the mother liquor whitish crystals (m.p. 116°, $[\alpha]_D -30.7^\circ$) separated out. Both the crystals were very easily soluble in acetone and alcohol and were hard to recrystallise them. LIEBERMANN'S and SALKOWSKI'S reactions of the preparations of melting points of 124° and 116° were compared as follows:

Liebermann's reaction

(M.p. 124°) light purple → light blue → light green → colourless → light green → bluish green (after a night over)

(M.p. 116°) greenish blue → green (the reaction was rather intense)

Salkowski's reaction*

(M.p. 124°) chloroform layer.....fluorescent reddish-yellow → faded
(The reaction (after a night over) → violet (the next day)
was intense)

sulphuric acid layer.....red → violet-red.

(M.p. 116°) chloroform layer.....fluorescent reddish-yellow → colourless
(The reaction (after a night over) → light violet (the next day).
was feeble)

Sulphuric acid layer.....red → violet-red.

The properties of the sterols and their derivatives are summed up as follows:

TABLE II. The properties of pelvesterol preparations separated from various seaweeds and their derivatives.

No.	Kinds of algae	Pelvesterol	Acetate	Propionate	Benzo-ate	Bromide of acetate
		M.p. $[\alpha]_D$	M.p. $[\alpha]_D$	M.p.	M.p.	M.p.
1	<i>Pelvetia W.</i>	122°–39.0°	118° –44.1°	104°	114°	132° (decomposed)
2	<i>Fucus e.</i>	122°–39.8°	118° –43.2°	103°	114°	
3	<i>Laminaria o.</i>	122° —	118° —	104°	114°	—
4	<i>Higikia f.</i>	122° –39.5°	118.5°–44.1°	105°–106° [α] _D = –43.1°	—	—
5	Long blades of <i>Alaria c.</i>	124°–41.6°	119.5°–46.9°	—	—	—
6	Seeding blades of <i>Alaria c.</i>	124°–41.3°	119.5°–46.5°	—	—	—
7	<i>Cystophyllum h.</i>	124°–41.5°	119.5° 46.6°	—	—	—
8	Fucoesterol	124°–38.42°	118–119° –43.8°	105–106°	120°	133°

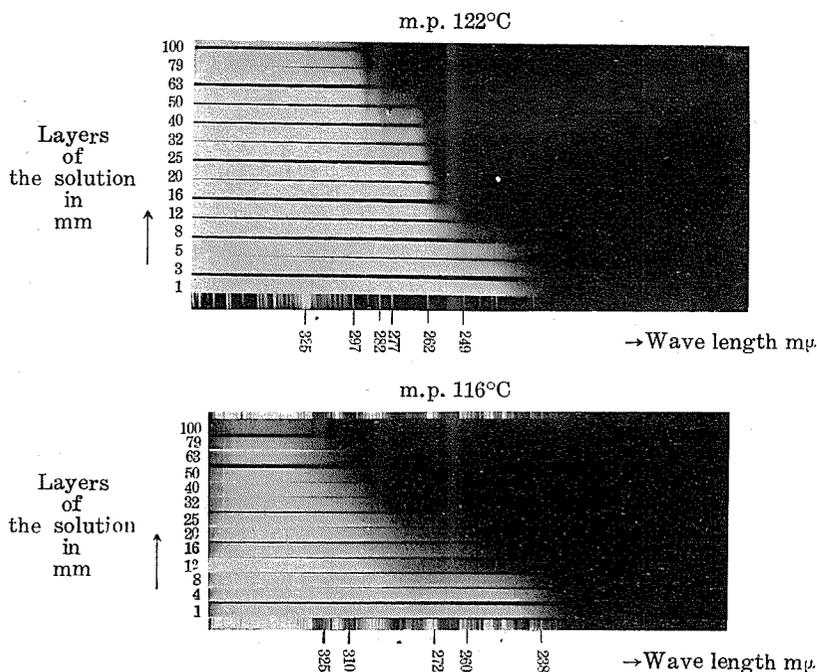
* With cholesterol and phytosterol it was difficult to separate the two phases after shaking up.

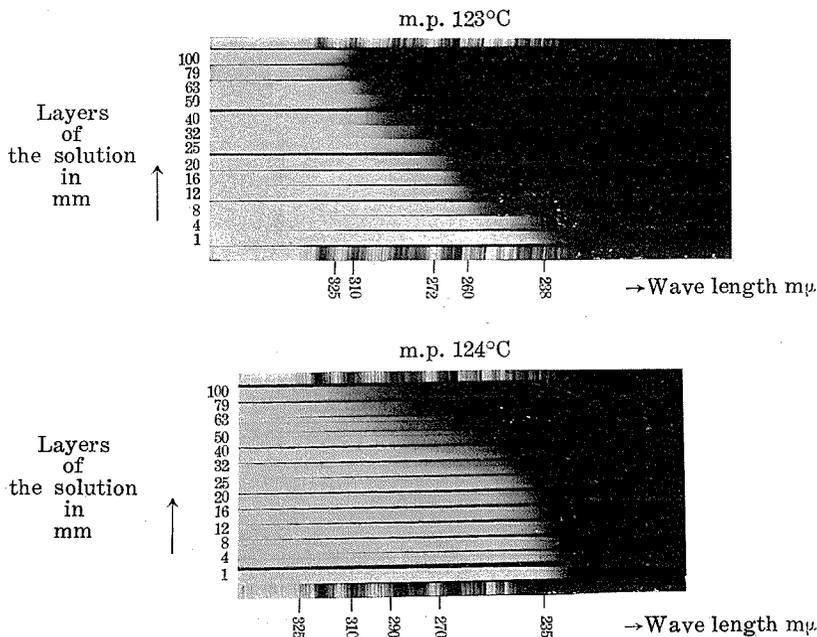
Although No.'s 1-4 of the second table almost coincided in their properties, a little difference was noted in the mixed melting tests of the crystals. On the contrary No's. 5-7 kept their melting-points constantly at 124° in the mixed melting tests. Perhaps No's. 1-4 contained a slight quantity of impurities.

When the sterol of m.p. 124° and its derivatives (fucosterol) obtained by HEILBRON and his collaborators are compared with the pelvesterol of m.p. 124° and its derivatives obtained by the author, rotatory power of fucosterol and its acetate seems to be too low. These numerical values are very approximate to those of rotatory power of the pelvesterol of m.p. 122° and their acetates obtained by the author. On this point BENGTSOON⁽¹⁾ raised a question, and HEILBRON and his collaborators⁽³⁾ carried out later reexaminations of their stanol.

The comparison of the absorption bands in the ultra-violet regions of the solutions of various preparations which differ in their melting-points is shown in figure 1.

Fig. 1. Absorption bands in the ultra-violet regions of the spectra of the solutions of various preparations.





The preparation of m.p. 122° was obtained from *Hijikia fusiforme* (HARV.) OKAM. and all the other preparations were obtained from *Cystophyllum hakodatense* YENDO. The solution of the sample of m.p. 116° was prepared by dissolving 8.5 mg. of the sample in 50 c.c. of ethyl alcohol; the solution of that of m.p. 122 was a M/1000 ethyl alcoholic solution; and the solutions of the preparations of m.p. 123° and 124° were M/100 ethyl alcoholic solution.

In the solution of the sterol obtained from *Hijikata fusiforme* (Harv.) OKAM. three narrow deep absorption bands near 2950, 2825 and 2730 Å, a broad absorption band with 2600 Å as its centre and a broader absorption band with 2500 Å as its centre were seen, but in the solutions of the preparations of m.p. 123° and 124° obtained from *Cystophyllum hakodatense* YENDO no slender absorption line was seen and in the latter hardly any absorption occurred. However, in the solution of the sample of m.p. 116° an absorption band of considerable extent was seen.

Of the purification of the pelvesterol separated from *Alaria crassifolia* KJELLM. and *Cystophyllum hakodatense* YENDO a description in detail will be given in another chapter of this paper.

B. Lipase-activating function of pelvésterol

From old times it has been well known that bile promotes the digestion of fats. It is thought to be the reason that fats are emulsified very well and minutely dispersed in particles when they come in contact with bile, consequently enlarging the space to be acted upon by the enzyme and so much promoting the activity. In other words, it is believed the reason why the lipase is activated by bile is not because bile directly activates the enzymic action of lipase, but indirectly favours by changing the physical state of fats. Similar explanations are given in the cases of lecithine and serum albumine.

On the other hand, in the field of pathology, opinions have come to be set forth in connection with cancer⁽³⁷⁾ and in other cases that a large or small quantity of cholesterol activates or restrains the action of lipase. But whether cholesterol acts directly or indirectly in the case has not yet been fully discussed to the conclusion.

The author was deeply interested in this matter and inquired into the problem of how the activity of lipase is influenced by the sterol which he has newly isolated.

1) Experiments

Samples. Lipase used was commercial steapsin (Lipase of the pancreas) and sterols used were the following preparations:

Pelvésterol. Various preparations from *Pelvetia Wrightii* YENDO, *Fucus evanescens* Ag., *Laminaria ochotensis* MIYABE, *Hijikia fusiforme* (Harv.) OKAM. *Alaria crassifolia* KJELLM and *Cystophyllum Hakodatense* YENDO that melt respectively at 122°.

Cholesterol. A preparation by Merck and Co., which melts at 142°.

Phytosterol. A preparation by Merck and Co., which melts at 136-7°.

Ergosterol. A preparation by Dr. SUMI of the Physico-Chemical Laboratory, which melts at 162°.

Natrium taurocholate. A preparation by Merck and Co., a water-soluble light brown powder.

Physical change of fats by the additions of sterols.

Various sterols were dissolved separately in triacetin and the surface tension of each solution was examined and on shaking up

with water the condition of each solution was examined. The results are compared as follows:

(a) Surface tensions of the triacetin solutions of sterols.

	Surface tension (Number of drops of 1 c.c.)
5 mg. pelvesterol (<i>Hijikia</i> f.) + 1 c.c. triacetin ..	57.00
5 mg. phytosterol + 1 c.c. triacetin	59.00
5 mg. cholesterol + 1 c.c. triacetin	60.00
1 c.c. triacetin	56.00

(b) Conditions of the triacetin solutions of sterols on shaking up with addition of water.

5 mg. pelvesterol (*Hijikia* f.) + 1 c.c. triacetin + 50 c.c. H₂O
Coagulation, clear solution.

5 mg. phytosterol + 1 c.c. triacetin + 50 c.c. H₂O
Slight coagulation, a little turbid solution.

5 mg. cholesterol + 1 c.c. triacetin + 50 c.c. H₂O
No coagulation, turbid solution.

1 c.c. triacetin + 50 c.c. H₂O
No coagulation, clear solution.

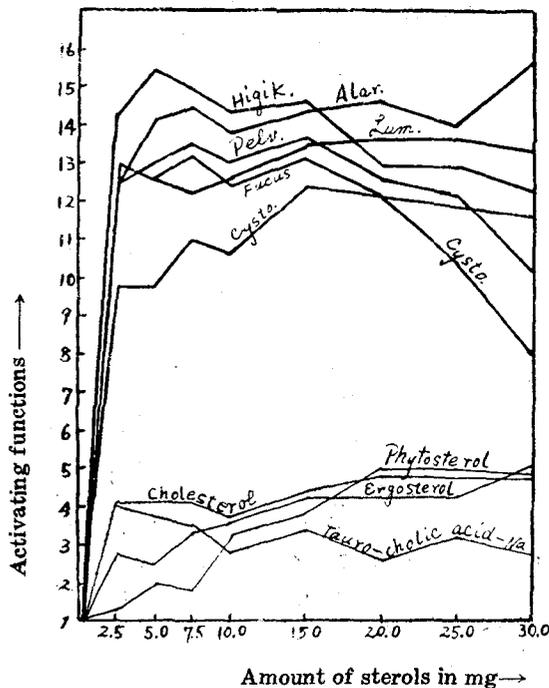
As shown above, when sterols were added, the surface tensions of fats decreased generally, and the number of oil-drops increased more and more in the order of additions of pelvesterol, phytosterol and cholesterol. This property may be observed clearer by the naked eye when the oils in which sterols are dissolved are shaken with water. In the solution containing cholesterol the oil-drops are dispersed very well and the solution is turbid (not so much as emulsified); in the solution containing phytosterol the oil-drops coagulate slightly and float on the surface; in the solution containing pelvesterol the oil-drops coagulate almost wholly and float on the surface and the solution turns clear. In the solution not containing any sterol, although the oil-drops are well dispersed when the solution is shaken up, they soon congregate and separate from water keeping it clear. From these results it may be assumed that cholesterol makes oil assume the most favourable physical condition for the action of enzyme and pelvesterol makes oil assume the most unfavourable. But the results of the experiments unexpectedly showed the contrary as described in the following paragraph.

Effects of sterols upon the digestion of fats

To examine what effects the presence of a sterol brings about when a fat is digested by means of lipase the following digestive liquid was prepared.

Preparation of a digestive liquid:—The required quantity of sterol (2.5, 5.0, 7.5, 10.0, 15.0, 20.0, 25.0 or 30.0 mg.) was placed in a triangular flask of 100 c.c. capacity equipped with a stopper, dissolved with an addition of 1 c.c. of ether, then 1 c.c. of triacetin, 5 c.c. of a buffer solution for hydrogen-ion concentration (at pH 8.0, prepared with phosphate manufactured by Kahlbaum and Co. and specified by Sørensen), 5 c.c. of an enzyme solution (0.5 g. of steapsin dissolved in 100 c.c. of water, shaken well and filtered transparent)

Fig. 2. Activating functions of various sterols for lipase.



and 40 c.c. of water were added. The flask was stoppered closely and the contents were mixed well by shaking up. On the other hand another digestive liquid containing no sterol was prepared as control.

Measurement. After the above described digestive liquid had

been placed in the thermostat at 37°C. for 3 hours, it was titrated with N/20 NaOH solution using phenolphthalein as indicator, then the digestive liquid for control was titrated as above. The quantity of the former titration minus that of the latter represents activating (in the case of a positive numerical value) or restraining function (in the case of a negative numerical value) of the sterol used.

The results of the experiments were as Table III and Fig. 2.

TABLE III. Activating functions of various sterols in different concentrations for pancreatic lipase.

Kinds of sterols	Quantity in mg. of sterol added	2.5	5.0	7.5	10.0	15.0	20.0	25.0	30.0	0
Quantities in c.c. of N/20NaOH required for titration of digestive liquids.	Pelvesteral									
	<i>Fucus</i> (M.p. 122°)	18.3	18.0	18.4	17.7	18.4	17.5	17.2	17.0	5.3
	<i>P.lv.</i> (" ")	17.5	17.9	18.4	17.9	18.4	17.3	17.0	15.1	4.8
	<i>Lam.</i> (" ")	18.3	18.0	17.6	17.9	18.6	19.0	19.2	18.9	5.3
	<i>Higik.</i> (" ")	19.5	20.6	20.2	19.6	19.8	18.1	18.1	17.6	5.2
	<i>Alar.</i> (" ")	18.5	20.0	20.0	19.7	20.2	20.5	19.8	21.1	5.8
	<i>Cysto.</i> (" ")	15.0	15.0	16.1	15.9	17.7	17.4	15.5	13.2	5.2
	Cholesterol	9.6	9.6	9.6	9.4	10.1	10.3	10.3	10.3	5.5
	Phytosterol	6.8	7.5	7.3	8.7	9.3	10.5	10.5	10.4	5.4
	Ergosterol	8.2	7.9	8.7	9.0	9.7	9.7	9.7	10.5	5.4
	Sodium taurocholate	9.7	9.4	9.2	8.6	9.1	8.4	8.9	8.6	5.7
Activating functions.	Pelvesteral									
	<i>Fucus</i>	13.3	12.7	13.1	12.4	13.1	12.2	11.9	11.7	
	<i>Pelv.</i>	12.7	13.1	13.6	13.1	13.6	12.5	12.2	10.3	
	<i>Lam.</i>	13.0	12.7	12.3	12.6	13.3	13.7	13.7	13.6	
	<i>Higik.</i>	14.3	15.4	15.0	14.4	14.6	12.9	12.9	12.4	
	<i>Alar.</i>	12.7	14.2	14.4	13.9	14.4	14.7	14.0	15.5	
	<i>Cysto.</i>	9.8	9.8	10.9	10.7	12.5	12.2	10.3	8.0	
	Cholesterol	4.1	4.1	4.1	3.9	4.6	4.8	4.8	4.8	
	Phytosterol	1.3	2.0	1.8	3.2	3.8	5.0	5.0	4.9	
	Ergosterol	2.8	2.5	3.3	3.6	4.3	4.3	4.3	5.1	
Sodium taurocholate	4.0	3.7	3.5	2.9	3.4	2.7	3.2	2.9		

By the examination of the results of the experiments, it will be seen that every sterol activates the lipase, but its ability differs somewhat according to its quantity. A most conspicuous feature is that pelvesteral have about three times stronger activating functions than cholesterol, phytosterol, ergosterol and sodium taurocholate.

Reason forbids one to consider the cause as lying with the larger dispersion of fat globules as in the case of bile, for if it were so, cholesterol must be the most powerful and pelvesterol must be less powerful than phytosterol. The experiments showed results contrary to the author's expectation and a question arose in his mind whether pelvesterol might have such a function as that of lipase. So experiments were carried out as described below in which pelvesterol was treated as if it were lipase.

As in the case of the preparation of the above described digestive liquids 5 mg. of pelvesterol were dissolved in 1 c.c. of ether; 1 c.c. of triacetin, 5 c.c. of a buffer solution and 45 c.c. of water were added; mixed well by shaking and the flask was placed in a thermostat at 37°C. for 3 hours. Besides, two other digestive liquids were prepared for control. One was prepared by dissolving 1 c.c. of triacetin in 1 c.c. of ether and adding 5 c.c. of buffer solution and 45 c.c. of water (A). The other was prepared by dissolving 1 c.c. of triacetin in 1 c.c. of ether and adding 5 c.c. of buffer solution, 5 c.c. of enzyme solution and 40 c.c. of water (B). The three liquids titrated at the same time showed the following results:

Digestive liquids	N/20 NaOH titration quantity in c.c.
Control liquids { A	3.1
B	5.3
Original liquids	3.1

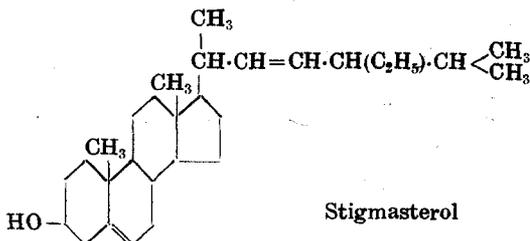
From these results it can be seen that pelvesterol has no such enzymic function as lipase at all.

2) Observations on the activating function

It is a most interesting fact that pelvesterol has a stronger activating function than other sterols and sodium taurocholate. The elucidation of its function must have an important significance in the studies of enzyme.

According to the researches of HEILBRON and his collaborators fucosterol (pelvesterol) is an isomer of stigmasterol and its molecular formula is $C_{29}H_{48}O$. According to Fernholz stigmasterol, as shown in P. 20, has two double bonds in a benzene nucleus and its side chain

while fucosterol, according to HEILBRON and his collaborators, has both its double bonds in benzene nuclei (their positions are not yet ascertained).



Further, ostreasterol that BERGMANN⁽²⁾ discovered in oysters and other mollusc is said to be an isomer of stigmasterol. But its saturated form coincides with sitosterol. Then all these three kinds of sterols have the same molecular formulae and two double bonds, only differing in the positions of them.

This is thought to have a very important significance in relation with the activating functions of these sterols, for it suggests that the activating the action of lipase, one of the direct actions of sterols has a very close relation with their unsaturation.

Upon observing the curves of lipase-activating functions of the sterols above described, it seems to the writer that the sterols coming from the similar sources form somewhat similar curves. But what is the cause of the dissimilarity of the pelvesterol preparations of which closer similarity was expected? It may not be due to the disparity of the conditions of fats at the time of digestion, but may be attributable to the purification of the pelvesterol. This point will be treated in the next paragraph.

C. Relation between the purification of pelvesterol and the curve of its lipase-activating function

As described in the preceding paragraph pelvesterol has special activating function for pancreatic lipase, but the curves of lipase-activating function of the pelvesterol that differed in the conditions of purification were not altogether similar.

So the author tried to carry out the purification of the sterol that was separated from the quite different two kinds of seaweeds as described in Chapter I. A. 5 and 6 till the curves of their lipase-

activating functions almost coincided. At first the sterol preparations which melted at 122° like those obtained before, were obtained and the curves of their lipase activating functions did not coincide. When they were acetylated, recrystallised from acetic anhydride,

TABLE IV. Comparison of the lipase-activating functions of the products obtained in the course of the purification of pelvesterol.

Pelvesterol from *Alaria crassifolia* Kjellm.

Kinds of samples	Quantity in mg. of sterol preparation added			2.5	5.0	7.5	10.0	15.0	20.0	25.0	30.0	0
	Quantities in c.c. of N/20NaOH required for titration of digestive liquids	A	M.p.	121°	16.9	18.0	17.8	18.3	18.3	18.0	18.3	—
122°				18.5	20.0	20.0	19.7	20.2	20.5	19.8	21.1	5.8
124°				18.5	18.9	18.2	19.5	16.2	14.5	15.2	16.3	5.4
B		M.p.	122°	15.1	15.2	13.5	15.7	16.3	17.1	16.6	16.6	5.4
			124°	15.0	15.3	14.6	15.7	13.8	12.6	13.8	14.3	5.9
Activating functions	A	M.p.	121°	11.5	12.6	12.4	12.9	12.9	12.6	12.9	—	
			122°	12.7	14.2	14.4	13.9	14.4	14.7	14.0	15.5	
			124°	13.1	13.5	12.8	14.1	10.8	9.1	9.8	10.9	
	B	M.p.	122°	9.7	9.8	8.1	10.3	10.9	11.7	11.2	11.2	
			124°	9.1	9.4	8.7	9.8	7.9	6.7	7.9	8.4	

Pelvesterol from *Cystophyllum hakodatense* Yendo.

Quantities in c.c. of N/20NaOH required for titration of digestive liquids	Pelvesterol	M.p.	122°	15.0	15.0	16.1	15.9	17.7	17.4	15.5	13.2	5.2	
			123°	15.0	16.7	16.3	17.4	14.2	15.0	15.3	17.2	5.4	
			124°	17.2	19.2	18.9	20.1	16.2	14.7	15.4	16.5	5.8	
	Acetate	M.p.	119.5°	10.1	12.5	12.2	12.9	12.4	12.3	13.8	—	5.8	
	Crystal	M.p.	80-86°	18.0	18.8	19.6	—	—	—	—	—	—	5.5
			116°	21.2	22.2	—	—	—	—	—	—	—	5.5
Activating functions	Pelvesterol	M.p.	122°	9.8	9.8	10.9	10.7	12.5	12.2	10.3	8.0		
			123°	9.6	11.3	10.9	12.0	8.3	9.6	9.9	11.8		
			124°	11.7	13.4	13.1	14.3	10.4	8.9	9.6	10.7		
	Acetate	M.p.	119.5°	4.3	6.7	6.4	7.1	6.6	6.5	8.0	—		
	Crystal	M.p.	80-86°	12.5	13.3	14.1	—	—	—	—	—		
			116°	15.7	16.7	—	—	—	—	—	—		

washed with acetic anhydride and liberated, their melting points rose higher than those of the samples obtained before and the curves of their lipase-activating functions came to coincide. When the absorption spectra of the solutions of these preparations were examined in

the ultra-violet regions, there were some solutions in which no absorption was seen, as described above. From the mother liquor of recrystallisation from acetic anhydride a sort of sterol was isolated which melted at a lower degree and possessed a strong lipase-activating function. Judged from the variation of the curve of its lipase-activating function and the changes of the absorption spectrum of

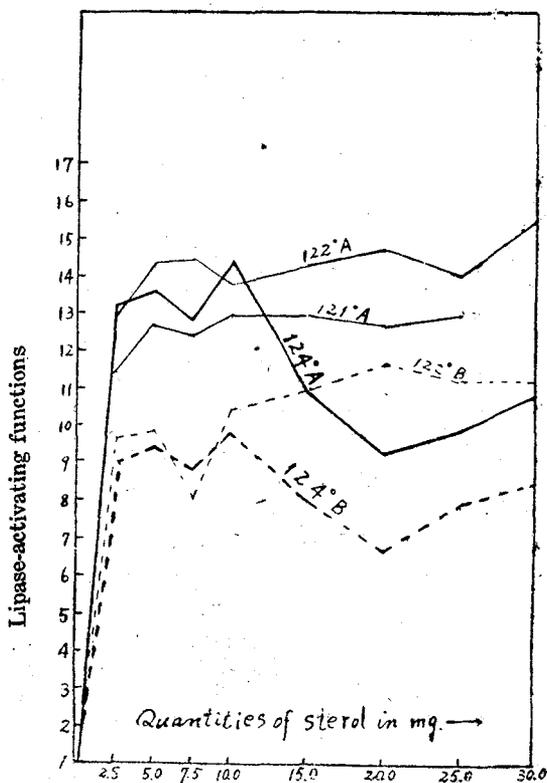


Fig. 3. Curve of the lipase-activating function of the pelvesterol from *Alaria crassifolia* Kjellm.

its solution this sterol was probably mixed in the sterol that melted below 124°. It is worthy of notice that despite the fact that the sterol isolated from the sporophyll of seaweeds almost coincided with that isolated from the blades of the seaweeds in melting-point, rotatory power and colour reaction, it differ solubility and the curves of lipase-activating function (Fig. 3).

As the lipase-activating functions of the products obtained in the course of the purification of *Alaria crassifolia* KJELLM. and *Cystophyllum hakodatense* YENDO were compared, the results were shown in Table IV. The methods of the experiments were quite the same as described in the preceding paragraph.

It may be seen that the degree of the purification of pelvesterol is not only connected with the strength of activation of lipase, but

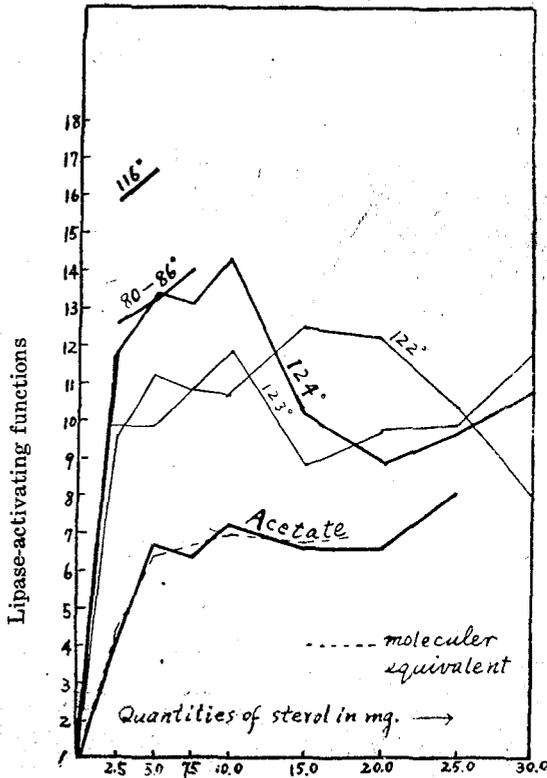


Fig. 4. Curve of the lipase-activating function of the pelvesterol and the acetates from *Cystophyllum hakodatense* Yendo.

also with the form of the curve of lipase-activating function. The attention must be paid to the fact that the pelvesterol from *Alaria crassifolia* KJELLM. of the A group forms a curve of higher position than those of the B group (Fig. 3). In connection with the pelvesterol obtained from *Cystophyllum hakodatense* YENDO the lipase-activat-

ing function of the acetate of the pelvesterol of m.p. 124° was examined. It was found that the highest portion of the curve of the activating function of the acetate was far lower than that of the original pelvesterol and that the right shoulder of the highest points was raised upwards (Fig. 4).

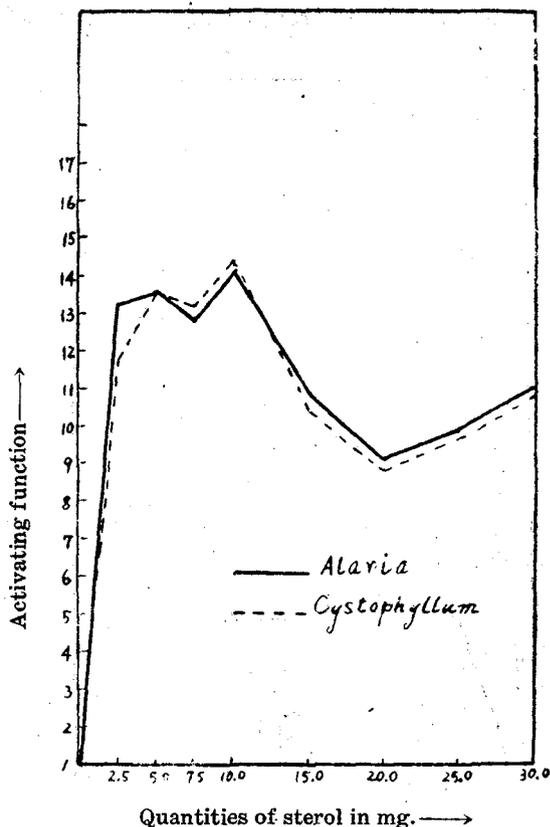


Fig. 5. Comparison of lipase-activating function of purified pelvesterol.

If the molecular formulae of sterol and steryl acetate be $C_{20}H_{48}O=412$ and $C_{31}H_{50}O_2=454$ as shown by HEILBRON and his collaborators, the molecular equivalents of steryl acetate that correspond with 5.0, 7.5, 10.0, 15.0, 20.0, 25.0, 30.0 and 35.0 mg. of sterol are 2.8, 5.5, 8.3, 11.0, 16.5, 22.0, 27.6 and 33.1 mg. The curve of the activating function of the steryl acetate as represented in dotted line presents a form only slightly different from that of the sterol (Fig. 4). This

remarkable effect that rises from acetylation suggests the important nature of the phenol group in relation with the lipase-activating function. It may be well supposed that the introduction of propyl, benzoyl and other groups which have respectively larger molecular weights than an acetyl group may exert remarkable effects on the curve of their lipase-activating function and it is clear enough that the phenol group of a sterol has a close relation with its lipase-activating function together with its unsaturated character mentioned in the preceding paragraph.

The strong function of lipase-activation of the substance which melted at 116° and that was produced in the course of purification of pelvesterol demands due attention. It makes one suppose the existence of such substances as this other than pelvesterol. These points will be treated later.

An examination was made of the melting-point of a mixture in equal amounts of the pelvesterol obtained from *Alaria crassifolia* KJELLM which melted at 124° and $[\alpha]_D = -41.6^{\circ}$ and that obtained from *Cystophyllum hakodatense* YENDO which melted at 124° and $[\alpha]_D = -41.5^{\circ}$. It was found to be 124° showing no change whenever. Further as the curves of their lipase activating functions were compared, they were found almost to coincide as shown in Fig. 5. Perhaps the forms of these curves represent definitely the properties of pure pelvesterol.

The forms of the curves of the lipase-activating functions of the pelvesterol preparations which were obtained in the course of purification and melted below 124° were all indefinite, and their differences were clearly to be seen. But the preparations of such grade of purification as of melting-point 122 – 124° showed only a slight difference in the mixed melting test as described above. The pelvesterol which melted at 122° was recrystallised from acetic anhydride, filtered off, washed with acetic anhydride and subjected to the process to liberate pelvesterol. The pelvesterol thus obtained not only had a higher melting-point, but produced many very striking changes in the curve of its lipase-activating function. From these changes the existence of a trace of some other substance which might be isolated by a treatment with acetic anhydride was suspected in the pelvesterol of melting-point 122° . Carrying out the isolation, a substance was obtained, which melted at 116° , had $[\alpha]_D = -30.7^{\circ}$ notwithstanding its being still impure, and possessed a remarkably

strong lipase-activating function. Considering that this substance was positive in both LIEBERMANN's and SALKOWSKI's reactions and from the viewpoint of the absorption spectra that were shown before it is thought to be a substance analogous to pelvesterol. After the elimination of the impurities the absorption spectra of pelvesterol was first known to be without absorption.

Further, what demands attention is the fact that a pelvesterol was isolated from the sporophyll of *Alaria crassifolia* KJELLM. which was of quite the same melting-point as one isolated from the blades of the same seaweed and made little difference with the same pelvesterol as to the rotatory power and colour reaction, but possessed a higher solubility and a lower lipase-activating function than the above mentioned pelvesterol. Consequently it may be well supposed that there is some intimate relation between the solubility and lipase-activating function of a pelvesterol. The explanation of this point is expected to become more and more clear as the chemical structure of the isomers of sterols are gradually elucidated hereafter.

II. Cramp and Narcotic Toxin

KÔZÔ KAWAKAMI and IWAO YAMAMOTO classified the toxins of fish liver oil into the three kinds of general fish oil toxin, cramp toxin and narcotic toxin, and reported in detail on the cramp and narcotic toxins existing in unsaponifiable matter, especially on the properties and methods of the isolation and the concentration of the cramp toxin. They thought the cramp toxin to be an essential constituent of fish liver oil and assumed that the constant existence of these toxins in fish liver oils might have arisen from some physiological necessity. For the elucidation of the question they made researches concerning the state of distribution of various fish liver oils, mammals liver oils and plant oils, but from the results did not reach any definite conclusion. M. MATUOKA⁽¹⁷⁾ formerly made detailed observations on the cramp actions of biosterin upon white rats and reported on the properties of it. In his report he stated that biosterin produced from *Enteromorpha* sp. showed itself positive to a considerable degree when he examined the presence of a cramp toxin in it.

The present author thought the presence of some substances like these in seaweed oils and after the examinations he found in all seaweed oils a substance which produced such cramp fits as were

observed and described in detail by M. MATUOKA, but in the oil of sporophyll of *Alaria crassifolia* KJELLM. he could find only a narcotic toxin.

A. Separation of toxins

The seaweeds used as materials were three kinds, *Alaria crassifolia* KJELLM. *Cystophyllum hakodatense* YENDO and *Laminaria ochotensis* MIYABE as described in the preceding chapter. They were dried in the sun, heated to 70–80°C., finely powdered by a pulveriser and digested with 80–90% alcohol. From each extract ether-soluble constituents were extracted with ether and the substance which precipitated with acetone was removed, and then crude fat was prepared from which unsaponifiable matter was separated.*

Materials	Quantities of materials in kg.	Yields of crude fats in g.	Yields of unsaponifiable matter in g.	Percentage of unsaponifiable matter in ratio to crude fats	
<i>Alaria crassifolia</i> Kjellm.	A	40.5	430	47 (from 293 g. of crude fat)	16
	B	15.5	150	8	5
<i>Cystophyllum hakodatense</i> Yendo	27.0	368	40	11	
<i>Laminaria ochotensis</i> Miyabe	20.0	200	14	7	

Materials	Yields of unsaponifiable matter in g.	Yields of liquid unsaponifiable matter in g.	Percentage of liquid unsaponifiable matter in ratio to whole unsaponifiable matter	
<i>Alaria crassifolia</i> Kjellm.	A	47	41	87
	B	8	5	62
<i>Cystophyllum hakodatense</i> Yendo	40	22	55	
<i>Laminaria ochotensis</i> Miyabe	15	12	80	

* The details of the isolation will be described in the following chapter.

N.B.: *Alaria crassifolia* KJELLM. was divided into two parts, namely the blades (A) and sporophyll (B) directly after it was collected and each part was dried and pulverised separately.

The yields of unsaponifiable matter separated were as shown in the above table.

In every case unsaponifiable matter was dissolved in a small quantity of absolute alcohol and sterol was separated by crystallisation. The mother liquor being a little concentrated and cooled with ice and salt, sterol was again crystallised and separated and alcohol was removed leaving a brownish-yellow, viscous, unsaponifiable matter behind.

B. Physiological actions of toxins

To know whether the liquid unsaponifiable matter be poisonous or not 0.5 g. of that of *Alaria crassifolia* KJELLM. (A) was injected hypodermically into a white rat of 65 g. weight as preliminary test. It developed the following symptoms.

Five minutes after the injection the rat was taken with a fit of cramps, raising and turning its fore limbs, stretching its neck and goggling its eyes, stretching its hind limbs to both sides and stretching its tail. After 17 and 22 minutes similar fits of convulsion occurred. After 28 minutes the whole body of the rat was taken with a fit of cramps so severe that the rat jumped and bounded about for 2 minutes and at last had trouble in walking, falling into a creeping posture. After 33 and 42 minutes severe fits again occurred and afterwards several intermittently. The rat broke down by degrees and was dead in 4 hours. When 0.5 g. of the liquid unsaponifiable matter of *Laminaria ochotensis* MIYABE was injected into a rat of 50 g. weight the rat was hardly affected in any way.

Test of the matter soluble in 80% methyle alcohol

The above-mentioned unsaponifiable matter was extracted with 80% methyl alcohol according to the method described by K. KAWAKAMI and I. YAMAMOTO. On removal of alcohol the residue was shaken up with additions of a little quantity of water first, then of some ether to separate the ethereal solution. On dehydration of the solution with anhydrous sulphate of soda the ether was removed to leave a liquid which was used for a hypodermic injection into a rat. The results were as follows:

Alaria crassifolia KJELLM. (A). From 16 g. of the liquid unsaponifiable matter 3 g. of the matter soluble in 80% methyl alcohol were obtained (19%). 0.5 g. of this matter was injected hypodermically into a white rat 63 g. weight. After 22 minutes the rat was taken suddenly with a violent fit of cramps, jumped and bounded about, fell on its side, lay on its back then raged about in the cage. After 27 minutes a similar violent fit occurred and afterwards some severe fits reoccurred intermittently. At last the rat was dead in 70 minutes.

Alaria crassifolia KJELLM. (B). On treatment of the liquid unsaponifiable matter with 100 c.c. of 80% methyl alcohol as above the alcohol was removed completely and emulsion of about 10 c.c. of water and an oily substance was obtained. Oil-drops that floated over the surface of the liquid were sucked up with a syringe and about 0.3 g. was obtained. About 0.2 g. of the emulsion was taken together with the oil-drops making up 0.5 g. all of which was injected into a rat of 79 g. weight (1). The 0.5 g. of the emulsion was injected into a rat of 65 g. weight (2) and 0.1 g. of the same into a rat of 80 g. weight (3).

The results were as follows:

- (1) After 3 minutes the rat was paralysed all over the body motionless (even turned on its back it continued the same posture). It was anaesthetic when it was pinched on the back, had several light fits of convulsion now and then after 30 minutes, but its complexion showed its sickness. It was dead in 50 minutes.
- (2) After 5 minutes the rat fell in a narcotic state and was almost insensible when pinched on the body, had a light convulsion after 20 minutes, could not recover its former posture when turned on its back. Its action was sluggish and it hardly wanted to move, only crouching. But by the next morning it had recovered vigour and kept on living.
- (3) After 10 minutes the rat was seized by a fit of narcosis over the hind half of the body and crouched almost motionless. It was insensible when it was trodden on the tail and scorched on the hind half of the body with a lighted cigarette (it was slightly responsive to stimuli fore half body). After 60 minutes it raised its fore limbs and had a slight fit of cramp, but at last recovered vigour by the next morning and kept on living.

Cystophyllum hakodatense YENDO. The above-described crude fat was extracted with 80% methyl alcohol without previous saponification; on removal of the alcohol saponification was carried out and the unsaponifiable matter which weighed 8 g. was separated. It was diluted to double volume with petroleum ether and 0.5 g. of it (this corresponded to 0.25 g. of the original) was injected into a rat of 100 g. weight (1). After extraction with 80% methyl alcohol the residue was saponified and 40 g. of unsaponifiable matter were separated, which was treated again with 80% methyl alcohol to give a trace (0.7 g.) of the soluble matter. It was dissolved in double volume of petroleum ether and 0.5 g. of the solution (this corresponded to 0.25 g. of the original) was injected into a rat of 105 g. weight (2).

(1) After 5 minutes the rat was attacked by a fit of cramps. After 10 minutes it was seized by a violent fit of cramps, jumped and bounded about, fell on its side or on its back and struggled in agony. Afterwards its cramps reoccurred intermittently and the rat was dead in 50 minutes.

(2) Nothing abnormal was to be seen.

Laminaria ochotensis MIYABE. Twelve grams of the liquid unsaponifiable matter were treated with 100 c.c. of 80% methyl alcohol as above described and were injected into a rat of 80 g. weight (1). Further, on removal of the methyl alcohol mentioned above, an extraction was carried out with ether and the remnant in the aqueous layer was concentrated and the whole quantity (0.85 g.) of it was injected into a rat of 64 g. weight (2).

(1) After 5 minutes the rat was taken with a fit of cramps. After 10 minutes it was seized by a severe fit of cramps, jumped and bounded about and squeaked, tumbling down at last to fall upon its back. After 20 minutes its complexion began to turn pale and some fits of cramps attacked it intermittently until death in 40 minutes.

(2) After 5 minutes a light fit of cramps occurred. After 8 minutes the rat was cramped as soon as it fell down stretching its body straight and after 28, 35, 38 and 40 minutes such fits of cramp occurred as made it sprang up. Afterwards more fits caught it. It was dead in 2 hours 15 minutes.

Other tests. In every case 0.5 g. of the residue of the extraction of the liquid unsaponifiable matter with 80% methyl alcohol was taken and injected into a rat for caution's sake. Hardly any abnormal symptoms appeared. Then after, 3.052 g. of the liquid unsaponifiable matter of *Alaria crassifolia* KJELLM. (A) were dissolved in ether and the solution was decolorised with Merck's animal charcoal, an attempt was made to recover as much quantity as possible; 1.72 g. were recovered, the percentage of loss being 56.3. When 0.5 g. of this substance was injected into a rat of 59 g. weight, the result was as follows:

After 16 minutes the rat was taken with a light fit of cramps and after 20 minutes a severe fit occurred. However, the rat had no fit of any importance afterwards and kept on living vigorously at last.

By distillation of 2.5 g. of the above-mentioned extract of *Alaria crassifolia* KJELLM. with 80% methyl alcohol under 3 mm. at 120-140°C., 15 g. of a light yellow liquid (A) was obtained, of which 0.2 g. was injected into a rat of 64 g. weight (A). The residue was dark brown and viscous. It was dissolved in an equal volume of petroleum ether and 0.5 g. of the solution (B) was injected into a rat of 52 g. weight (B). When (A) was allowed to stand, colourless crystals separated out. The crystals were very complex solids of which one face made a long hexagon having two short sides. A part of the crystals was washed with petroleum ether, and 15 mg. of it were dissolved in 0.5 c.c. alcohol and the solution (C) was injected into a rat of 65 g. weight (C). The results were as follows:

(A) After 8 minutes the rat was taken with a fit of cramps.

After 12 minutes it was attacked by such a severe fit of cramps that it leaped about and tumbled down on its back.

After 20 minutes a similar fit occurred again. After 25 minutes it was attacked by such a severe fit that it sprang up throwing its neck backwards and raged about in the cage. After 30 minutes it fell on its side and was cramped; breaking down gradually was dead at last in 2 hours and 10 minutes.

(B) After 30 minutes the rat was seized by a light fit of cramps and afterwards it was seized by several light fits now and then, but kept on living vigorously after all.

(C) There was no abnormality to be seen.

saponifiable matter obtained from the blades of *Alaria crassifolia* KJELLM. was decolorised with animal charcoal its quantity was reduced by half and its poisoning power was also reduced by half. The toxins were perhaps considerably absorbed by the animal charcoal. But when the unsaponifiable matter was distilled *in vacuo* more powerful poisonous constituents were obtained. The poisonous constituents may be obtained almost wholly by the direct extraction of crude fats with 80% methyl alcohol.

It is very interesting that these poisonous constituents occur in the seaweeds as in fish liver-oils. The chemical properties of the concentrates of these constituents will be described in the next chapter.

III. The Liquid Constituents of Unsaponifiable Matter of Fats

RUSSEL-WELLS⁽²¹⁾ stated that the amounts of fat and unsaponifiable matter contained in the seaweeds has close relation with the situations and the depths of the places where seaweeds grew and that the longer the seaweeds were exposed in the air, the larger the amounts of the two substances were. HEILBRON, PHIPERS and WRIGHT⁽⁷⁾ extracted the dried powder of *Fucus vesiculosus* with 95% cold alcohol, shook up the extract with an addition of petroleum ether and saponified. On separating the unsaponifiable matter dissolved it in methanol and carried out a fractional crystallisation, and from the mother liquor of fucosterol that first crystallised out separated hentriacontan ($C_{31}H_{64}$) which showed a melting-point of 67°C. This constituent had been separated often from higher plants, but not previously from seaweeds. Further, Heilbron and his coworkers reported that they obtained a strongly stinking violescent-grey liquid which they supposed to be a terpene by the steam distillation of the mother liquor.

It was stated in the preceding chapter that there occurred cramp and narcotic toxins with strong physiological actions, although slight in quantities, in the unsaponifiable matters of the fats obtained from *Alaria crassifolia* KJELLM., *Cystophyllum hakodatense* YENDO and *Laminaria ochotensis* MIYABE. In this descriptions somewhat in detail will be given of the properties of the concentrates of these

poisonous constituents and the existence of some hydrocarbons separated in addition to these substances.

A. Separation of the liquid unsaponifiable matters

The seaweeds employed as materials were the blades of the above mentioned *Alaria crassifolia* KJELLM. After drying in the sun they were heated to 70–80°C. and powdered finely with a pulveriser. Every 3 kg. of the powder were extracted with 3.5 l. of 80–90% alcohol on a boiling water-bath for 3–4 hours, the extract was filtered while hot and the residue was extracted with 2 l. of alcohol in the same manner as before. The filtrates were allowed to stand until cool, then mixed together, filtered and allowed to stand. On removal of the crystals of mannit and other impurities the filtrate was concentrated. After adding a little quantity of water, then shaken with some ether. The ethereal solution was separated, dehydrated with anhydrous sulphate of soda, and the ether was removed. By repetition of the process 651 g. of a syrup (including 181 g. of water) were obtained from 40.5 kg., the total amount of the seaweed powder. By adding 10–20 volumes of acetone to the syrup, phosphatide (below) was separated and on expulsion of the solvent from the solution by concentration, 430 g. of crude fat containing a large quantity of brown pigment were obtained. When 293 g. of the crude fat were taken, added 500 c.c. of 10% alcoholic solution of KOH, saponified warming on a water-bath for 1 hour, an amine-like smell evolved in this case. The vapour turned red litmus-paper blue (*vide* the part of the bases below). On removal of the alcohol the solution was poured into water and on addition of a large quantity of ether was shaken up completely to transfer the unsaponifiable matter into the ether. The ethereal solution had a yellowish-brown coloration. After dehydration with anhydrous sulphate of soda the ether was removed and a reddish-yellow solid weighing 47.0812 g. (equivalent to 16% of crude fat) was obtained.

The unsaponifiable matter thus obtained was dissolved in a small quantity of absolute alcohol and a fractional crystallisation was carried out. At first a large quantity of pelvesterol crystallised out and it was separated. Then by cooling with ice and salt the mother liquor was crystallised and on removal of the crystals the remnant liquid was concentrated till the alcohol was completely expelled to

obtain residue weighing 33.2789 g. (71% of the whole unsaponifiable matter). This residue was allowed to stand overnight cooled at -10°C ., and by separating the crystals (about 2 g.) that separated out, about 31 g. of liquid unsaponifiable matter (66% of the whole unsaponifiable matter) were obtained.

Treatment of liquid unsaponifiable matter. Sixteen grams of the above mentioned liquid unsaponifiable matter were taken and worked with a glass rod adding 80% methanol little by little by which extracted solution was separated. All the residue was dissolved in petroleum ether and the solution was shaken up with an addition of 90% methanol little by little. Finally, the liquid unsaponifiable matter was fractionated into 3 parts as follows:

80% Methanol extracts	3 g. (19%)
90% Methanol extracts	5 g. (31%)
Petroleum ether extracts	8 g. (50%)

B. Properties of the extracts with various kinds of solvents

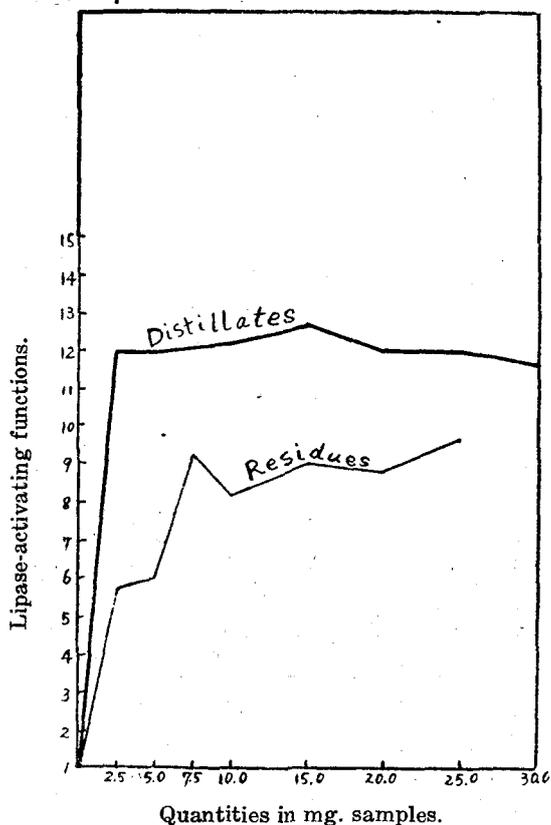
Part dissolved in 80% methanol. Two and five-tenths grams of the sample were taken and distilled at $120-140^{\circ}$ under reduced pressure of 3 mm. whereupon 1.5 g. (60%) of a light violet, peculiar

TABLE VI. Lipase-activating function of the distillates and the residues.

Sample in mg.	Distillates under 15 mm. and at $120-140^{\circ}\text{C}$		Residues of the distillates	
	Titration no. in c.c. of N/20 NaOH	Lipase-activating function	Titration no. in c.c. of N/20 NaOH	Lipase-activating function
2.5	18.0	12.0	11.8	5.8
5.0	18.0	12.0	12.0	6.0
7.5	18.1	12.1	15.3	9.3
10.0	18.2	12.2	14.1	8.1
15.0	18.7	12.7	15.2	9.2
20.0	18.0	12.0	14.8	8.8
25.0	18.0	12.0	15.6	9.6
30.0	17.0	11.7	—	—
0	6.0		6.0	

beach-smelling oily substance were obtained. The residue was dark brown and viscous. The distillate had, as stated in the preceding chapter, a strongly poisonous character and moreover a strong specific function of activating lipase. That character was examined by the same method as described in Chap. I and the results were obtained as shown in Table VI and Fig. 6.

Fig. 6. Lipase-activating function of the concentrates of poisonous constituents.



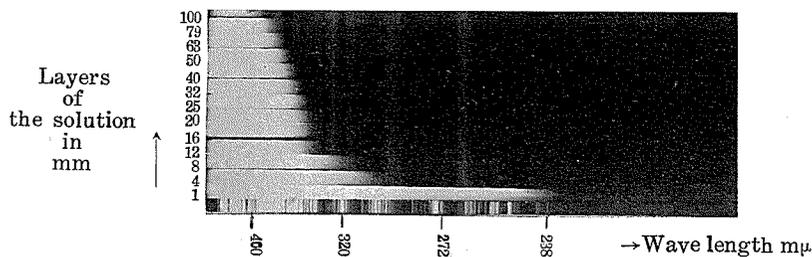
As seen in Fig. 6, the curve of lipase-activating function of the distillates had almost nominal curvature suggesting that it had hardly any relation with the concentration of the distillates. In the test of LIEBERMANN'S reaction, brown colour developed and in the test of SALKOWSKI'S reaction, the sulphuric acid layer turned crimson and the chloroform layer brown.

The elementary compositions of the distillates were determined as follows:

Quantities of the substances (mg)	CO ₂ (mg)	H ₂ O(mg)	C%	H%	O%
4.320	11.365	3.735	71.75	9.67	18.58
3.840	10.140	3.420	72.02	9.96	18.02
Average value ...	—	—	71.89	9.81	18.30

The distillates had the iodine value of 147 and when the absorption spectrum in the ultra-violet region was examined it was, as shown in Fig. 7, similar in some respects to that of sterol shown in Fig. 1 in Chap. I.

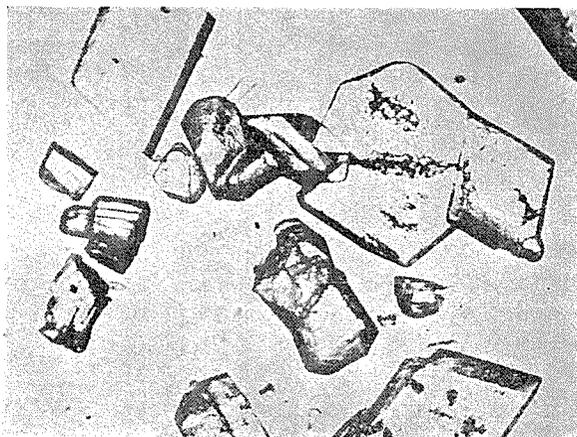
Fig. 7. The absorption spectrum of distillate.



Spectrum : 0.104 g. of the sample was dissolved in 20 c.c. of ether, diluted to 10 volumes and photographed using a hydrogen discharge tube as light source.

When the liquid was allowed to stand for many days, the crystals melting at 149°C. as shown in Fig. 8 were obtained. But these crystals had no poisonous character nor lipase-activating function at all.

Fig. 8. Crystals produced from the distillate (m.p. 149°C.)



(eight-fold enlarged)

The elementary composition of the crystals was determined as follows:

Quantity of the substance (mg)	CO ₂ (mg)	H ₂ O(mg)	C%	H%	O%
1.505	3.745	1.065	67.87	7.92	24.22
C ₇ H ₁₀ O ₂ (empirical formula)	—	—	66.66	7.93	25.39

As the sample was scanty a complete research was unable to be carried out.

Part dissolved in 90% methanol. This part had only a slightly poisonous character, but as its trichlorantimony reactoin was strong a fair amount of vitamin A was thought to exist. The lipase-activating function was recognized also to a slight degrees.

Part dissolved in petroleum ether. This part made up 50% of the liquid unsaponifiable matter. As it contained a fair amount of brown pigment it was dissolved in ether and decolorised with bone charcoal, and on removal of the ether it turned a light yellow oily liquid. The reflective index of the liquid was $n_D^{20} = 1.4681$ and the iodine value (by WIJS' method) 190 (at the estimation of the iodine value a white precipitate occurred). A part of the liquid was taken, dissolved in 3 volumes of acetone and cooled with ice and salt for several hours, but no crystals were produced. Perhaps the liquid consisted almost wholly of hydrocarbon and contained a higher unsaturated compound. To see whether a squalene-like substance existed or not, a small quantity of the sample was dissolved in ether and dried hydrochloric acid gas was introduced into the solution, but no white precipitate (squalene hexahydrochloride) resulted at all.

According to TUJIMOTO⁽³³⁾ a white turbidity occurs in the trichlorantimony reaction while a hydrocarbon of the terpene series exists; according to LEVINE and RICHMAN⁽¹⁶⁾ there occurs no white precipitate if acetic anhydride is added previously. A small quantity of the sample was dissolved in chloroform and added a chloroform solution of trichlorantimony whereupon the liquid instantly turned blue, then changed successively to bluish green and to purple producing a white precipitate in the process. But when trichlorantimony was added, after acetic anhydride had been previously added, there occurred no white turbidity and a clear coloration resulted turning blue, dark indigo, reddish-brown and reddish-purple in turn. From these reactions it was inferred that there was an unsaturated hydro-

carbon of the terpene series in the sample. When the sample dissolved in methanol was allowed to stand, transparent oily substance separated out of the solution.

Hydrogenation test. When 1.2 g. of the above mentioned sample were dissolved in 10 c.c. of absolute alcohol and hydrogenation was carried out employing platinum black as a catalyst the sample absorbed hydrogen easily as shown in the following figure and separated crystals.

- I. First crystal 0.25 g., m.p. 37.5–38°
- II. Crystal from the mother liquor of the above
a little, m.p. 37°
- III. Concentrate of the remnant liquid of the mother liquor ..
0.814 g.

When the crystals of I and II were recrystallised from acetone, they turned into crystals which melted at 41.0–42.5°, 42.0–42.5° and 37.5° (crystallised from under 90% alcohol) and by repeated recrystallisation, crystals with constant melting-points at 40.5–41.0° and 37.5° were obtained.

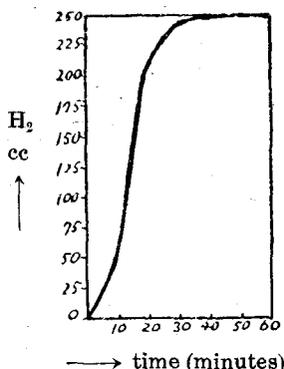
The elementary composition of the crystals which meted at 41° was determined as follows:

Quantities of the substances (mg)	CO ₂ (mg)	H ₂ O(mg)	C%	H%
3.570	11.065	4.625	84.53	14.49
2.585	8.015	3.415	84.56	14.78
Average value	—	—	85.55	14.64
C ₂₁ H ₄₄	—	—	85.15	14.86

According to the literature⁽²⁰⁾ the melting-point of heneicosane (C₂₁H₄₄) was 41° which coincides exactly with that of these crystals. The crystal with melting-point of 38° coincide with eicosane (C₂₀H₄₂).⁽²⁰⁾

When the concentrate (0.8140 g.) of the remnant liquid of the mother liquor from which the crystals had been separated was placed in a refrigerator under -10°C. overnight it turned transparent producing no crystals. The liquid was hardly soluble in glacial acetic acid, acetic anhydride and methanol, and on long

Fig. 9. The hydrogenation of the part dissolved in petroleum ether.



standing separated a transparent oily substance. The index of refraction of this substance was found to be $n_D^{20} = 1.4385$. Considering its solidifying-point it is certain that the substance was not a normal compound.

Bromination test. Six grams of the sample were taken to be dissolved in 2 c.c. of ether and while the solution was cooled with ice some glacial acetic acid solution of bromine was dropped in until the solution turned yellowish-brown (it took 2 hours); then the solution was allowed to stand overnight in a refrigerator at -7°C ., and the white precipitate produced was filtered off, washed well with ether and dried. The yield was 2.4 g. (40%). When the precipitate was heated it turned yellow at 170° , brown at 200° , dark brown at 220° , black at 222° and melted at 223° .

When 2 g. of this bromide were treated with hot benzol, the most part of it dissolved.

- I. Bromide soluble in hot benzol—14 g. Melted at $230\text{--}232^\circ$.
- II. Bromide insoluble in hot benzol—0.6 g. Decomposed evolving a gas at $240\text{--}241^\circ$.

Treatment of the bromide soluble in hot benzol. One gram of this bromide was suspended in glacial acetic acid and on addition of zinc dust the mixture was boiled for 40 minutes on a water-bath and filtered; the filtrate was shaken up with an addition of water and ether, then the ethereal layer was separated, washed well with water, dehydrated with anhydrous sulphate of soda and on removal of the ether 0.2 g. of an oily substance was obtained. A part of the substance was dissolved in alcohol to be hydrogenated; absorbing hydrogen, it quickly produced crystal of melting-point 41° . When this crystal was melted mixed with the crystal which was obtained in the above-described hydrogenation test and had its melting-point at 41° there occurred no lowering of the melting-point, accordingly both the substances were thought to be the same. The iodine value estimated was 269, although it was not exact owing to the small quantity of the sample. So the original hydrocarbon considered perhaps to be $\text{C}_{21}\text{H}_{38}$ (the calculation number of the iodine value 262).

Bromide insoluble in hot benzol. The estimation of the bromine content showed the following result:

Sample 32.7 mg. AgBr 53.5 mg. Br 69.7%.

Five-tenths gram of this sample was debrominated in the same manner as above described and a very small quantity of an oily substance was obtained. When this substance was hydrogenated there occurred a trace of crystal. Its melting-point was 37.5° and when it was melted together with the crystal which was obtained in the above-described hydrogenation test and had the melting-point at 37.5° there was recognized no decrease in the melting-point. Therefore it is inferred that the saturated substance may be $C_{20}H_{42}$ and the original unsaturated substance may be $C_{20}H_{34}$ (the calculation number of bromine contained in the bromide is 70%).

Solution of the bromide soluble in ether. By means of an aqueous solution of thiosulphate of soda the excessive bromine was removed from the above-mentioned filtrate from which the bromide insoluble in ether had been removed, and the filtrate was washed with water, dehydrated with anhydrous sulphate of soda, concentrated to remove the ether and treated with petroleum ether (distilled under $40^{\circ}C.$) whereby the most part dissolved except a minute quantity remaining insoluble. On removal of the insoluble substance the solution was concentrated, dissolved in glacial acetic acid, added zinc dust and debrominated in the same manner as described above whereupon 2 g. of an oily substance were obtained. This substance was known to contain no bromine by the examination of the colour of the flame. Its iodine value was 89.8 and its index of refraction $n_D^{20} = 1.4420$. When 0.5 g. of the liquid was dissolved in absolute alcohol and hydrogenated in the same manner as described above, the liquid absorbed hydrogen very easily and produced a very minute quantity of crystal (m.p. $34-35^{\circ}$). After filtrating off the crystal, the solution was concentrated and the alcohol was removed. The concentrate was similar to the liquid obtained by the above-mentioned hydrogenation test in that it produced no crystals though it was allowed to stand in a refrigerator under $-10^{\circ}C.$ for 2-3 days. It had the property of being hardly soluble in acetic anhydride and methanol, and the most part of it was distilled at about $160^{\circ}C.$ and under 10 mm. Its constitution was found to be as follows:

Quantities of the substances (mg)	CO ₂ (mg)	H ₂ O(mg)	C%	H%
4.270	13.30	5.63	84.94	14.65
3.515	10.99	4.71	85.27	14.89
Average value	—	—	85.10	14.77
C ₁₈ H ₃₈ (calculation number)	—	—	85.04	14.96

Its index of refraction was $n_D^{20} = 1.4370$ which is almost the same as that of the liquid obtained before. According to the literature the distillate is likely to be pristane ($C_{18}H_{38}$) and therefore the original unsaturated hydrocarbon is inferred to be $C_{18}H_{36}$ (the calculation number of the iodine value of $C_{18}H_{36}$ is 100.7). But the iodine value of the distillate is thought to be somewhat too low which may be because there was mixed a small quantity of some substance such as pristane from the first.

C. Considerations

Dried and pulverised *Alaria crassifolia* KJELLM. was digested with 80–90% alcohol and the infusion was concentrated and the matter extracted from it with ether was separated. On removing the ether the extract was treated with acetone, and on removal of phosphatide⁽²³⁾ the acetone solution was concentrated to obtain a crude fat containing brown pigment. The fat was saponified and unsaponifiable matter corresponding to about 16% of the crude fat was separated. The unsaponifiable matter was dissolved in a small quantity of alcohol and a fractional crystallisation was carried out. After removing the pelvesterol which crystallised out first and the crystal which separated out on cooling the mother liquor with ice and salt, the remainder was fractionated at last into an about 30% crude sterol and an about 70% liquid part.

The liquid part was fractionated into 3 parts that soluble in 80% methanol, soluble in 90% methanol and that soluble in petroleum ether and the constituents of the respective parts were examined. A cramp toxin which had the same properties as those which were discovered by K. KAWAKAMI and I. YAMAMOTO in the unsaponifiable matter of liver oils was found to be contained in the part soluble in 80% methanol. Researches were made as to its properties, but as the yield was scanty a full elucidation of them could not be attained after all.

The elementary composition of the distillate of the material was near $(C_6H_{10}O)_n$ and the iodine value of the distillate was 147. As the absorption spectrum was examined in the ultra-violet region there were some points similar to those of the above-described sterols. The distillate had a specific lipase-activating function like sterols. According to the literature⁽³⁰⁾ some of the phenanthren derivatives

have cramp and narcotic function. Taking these points into consideration we are inclined to think the distillate may have close relation with these substances. From the distillate there were separated out a slight quantity of crystals which melted at 149° and had the elementary composition of $(C_7H_{10}O_2)_n$. It is clear that the crystals were a substance mixed in the poisonous substance as they had neither poisonous character themselves nor lipase-activating character as the liquid part.

When the part soluble in 90% methanol was examined, it was found to give a strong trichlorantimony reaction. Perhaps this was due to the presence of vitamin A.

Further, when the part soluble in petroleum ether was examined, it was found to occupy about 50% of the whole liquid part and to consist for the most part of an unsaturated hydrocarbon. When it was treated with WIJS' reagent a white precipitate was formed showing the presence of a higher unsaturated compound in it. By the trichlorantimony reaction the presence of a hydrocarbon of the terpene series was recognized, but the reaction to squalene was negative.

When a hydrogenation was carried out there was found a small quantity of crystals which melted at 41° coinciding with heneicosane ($C_{21}H_{44}$) and some which melted at 38° coinciding with eicosane ($C_{20}H_{42}$). Considered from the results of the bromination test their originals corresponded to $C_{21}H_{38}$ and $C_{20}H_{34}$. The mother liquor of the crystals seemed to consist of an unsaturated compound $C_{18}H_{36}$ in the main.

According to the researches of TUJIMOTO and TOYAMA⁽³⁴⁾ the hydrocarbons pristane ($C_{18}H_{38}$) and zamene ($C_{18}H_{36}$) seem to be broadly distributed in shark liver oil always accompanying squalene, but notwithstanding their comparatively abundant presence in this seaweed oil the presence of squalene was not recognized there.

Although, as to the presence of the unsaturated hydrocarbons $C_{21}H_{38}$ and $C_{20}H_{34}$ more researches are necessary, the fact that a fairly large quantity of heneicosane ($C_{21}H_{44}$) is produced by hydrogenation is certain enough. As the presence of these unsaturated hydrocarbons is not well known even in fish oils these compounds will surely attract general interest as newly discovered ones.

IV. Phosphatide⁽²⁴⁾

According to the literature published up to date it is known that phosphatide exists with sterol in every tissue-cell and that its amount is comparatively large in the tissue-cell of the parts of vigorous vitality. In modern researches on metabolic activity of the tissue the properties and the amount of the fatty acid which is looked upon as a specific constituent of phosphatide are focuses of interest and supposition.

However, there has been nothing reported hitherto on phosphatide in the seaweeds. Although some writers have reported on the existence of certain organic or inorganic phosphates⁽⁴⁾ in the seaweeds, no one has yet pointed out the presence of phosphatide there.

Kylin⁽¹³⁾ recognized that the phosphorous reaction was positive in the aqueous infusion of most seaweeds and reported that the reaction was especially remarkable in the infusions of the young blades of *Ascophyllum* and *Fucus* of *Phaeophyceae*. Further, TADOKORO, ABE and YANASE⁽²⁹⁾ reported that as they had estimated the phosphates of the ash of *Laminaria longissima*, *Undaira pinnatifida* (Harv.) Suring, *Enteromorpha* sp. and *Iridaea* the ratio of the amounts of phosphates to those of the ash had been remarkably diverse.

As often stated above a substance which was precipitated with acetone was recognized in the crude fat of the blades of *Alaria crassifolia* KJELLM.; so it was separated and tested for its phosphorus reaction. The reaction was remarkable, so some research of the properties of the substance were carried out with the results to be given in the following paragraphs.

A. Separation of Phosphatide

The blades of *Alaria crassifolia* KJELLM as mentioned above were used as materials.

To every 3 kg. of the powdered materials 3.5 l. of 80-90% alcohol were added to carry out a digestion for 3-4 hours on a water-bath, filtered the extract while hot, digested the residue with 2 l. of alcohol and filtered the extract as before. On cooling the filtrates were mixed together and filtered. After removing a large amount

of the crystals of mannite that separated out and other impurities the filtrate was concentrated and shaken up with ether after addition of a small quantity of water; the ethereal solution was separated and on dehydration with anhydrous sulphate of soda the ether was removed. By this procedure 651 g. of crude fat (still containing a fair amount of water because of incomplete dehydration) were obtained from the powdered materials. When 10-20 volumes of acetone were added to the fat, phosphatide appeared as a voluminous brown precipitate. It was separated centrifugally, dissolved again in ether and precipitated with acetone as before. After repeating this process until the stain of acetone by pigment became slight, phosphatide was taken out and dried in a desiccator. The yield weighed 40 g.

For further purification the product was first dissolved in petroleum ether (though with difficulty it dissolved almost completely), filtered, and the filtrate was precipitated with acetone; the precipitate was dissolved in a small quantity of ether and the solution was added with stirring to a large quantity of absolute alcohol whereupon the most part precipitated as a brown glutinous substance to the bottom of the vessel and the solution turned pale yellow and transparent. By decantation the mixed alcohol and ether solution was removed and the precipitate was subjected repeatedly to the process of solution in ether and precipitation with absolute alcohol until the solution was hardly stained. The precipitate was dissolved in ether and the solution was precipitated with acetone. The precipitate was separated and dried in a vacuum desiccator to get a yield of about 20 g. When powdered, the dried precipitate was pale yellowish-green.

When all the alcohol solutions were mixed together and a saturated alcohol solution of cadmium chloride was added there was formed a precipitate which was separated and dried *in vacuo* to get a yield of about 4 g.

B. -General properties

The separated and purified substance of 20 g. yield mentioned above was soluble in ether and petroleum ether, insoluble in acetone and absolute alcohol and its phosphorus reaction was remarkable. It closely resembled cephaline. The substance of 4 g. yield separated as a cadmium salt was soluble in ether, petroleum ether and absolute alcohol, and insoluble in acetone and its phosphorus-reaction was

remarkable. It closely resembled lecithine. A description will be given of the cephaline-like substance paragraphs.

This substance was very hygroscopic. When its ethereal solution was shaken up with water, the liquids turned colloidal and their boundary surface did not appear easily, unless the volume of ether was far larger than that of water. The ethereal solution thus separated could not be easily dehydrated with anhydrous sulphate of soda. The aggregate weight of phosphatide and crude fat separated from the 651 g. crude fat syrup mentioned above was 470 g., so if one looks upon the difference 181 g. as the weight of water, that weight would be heavier than that of the phosphatide by about four times and a half.

In the air the substance absorbed moisture and not only turned black, but degenerating, produced a substance insoluble in ether. It seemed to be sensitive to sunlight, for when a desiccator containing a weighing tube in which the sample had been put, was placed on the experiment table, the sample faded to yellow on the light side facing the window but remained pale yellowish-green as ever on the shady side. The melting-point of the substance was generally 175–180°. The other properties were as follows:

- Iodine value.* Sample (a) 0.4375 g., (b) 0.4202 g.,
Iodine (a) 275.28 mg., (b) 256.04 mg.,
Iodine value (a) 62.9, (b) 62.0, average 62.4.
- Phosphorus.* Sample 1.000 g., $\text{Mg}_2\text{P}_2\text{O}_7=0.0925$ g. $\text{P}=0.0284$ g.,
2.84%.
- Total nitrogen and amino nitrogen.* Sample 0.02 g. Total nitrogen 1.30% (by the micro Kjeldahl method).
Amino nitrogen 1.39% (by the micro Van Slyke's method).
- N : P.* $\text{N} : \text{P}=0.092 : 0.091=1 : 1.$
- Iodine.* Sample (a) 0.300 g., (b) *ditto*. To the sample was added 5 c.c. of 20% NaOH and burned. The residue was extracted with 95% alcohol and on removal of the alcohol the extract was dissolved in a small quantity of water and the solution was slightly acidified with 2N. H_2SO_4 (after neutralisation using methyl orange as indicator, one more drop added), oxidised with 1 c.c. of saturated bromine water,

boiled to drive off the excessive bromine, added a small quantity of solution of KI and titrated with N/1000 $\text{Na}_2\text{S}_2\text{O}_3$ using starch paste as indicator, thus obtaining the following results: (a) 0.32 c.c., (b) 0.28 c.c., average proportion of iodine 0.0019%.

Reducing power. A small quantity of the sample was hydrolysed with hydrochloric acid and the solution was neutralised with NaOH and when Fehling's reaction was tested, it was slightly positive.

Hydrolysis I

To 5 g. of the sample were added 300 c.c. of 1.2% baryta water to be hydrolysed on the water-bath for 4 hours. After cooling the solution was divided by filtration into residue (A) and filtrate (B).

Detection of fatty acids. The residue (A) was suspended in water and the mixture was shaken up with additions of hydrochloric acid and ether. The aqueous layer became pale yellow and transparent, and the ethereal layer was stained brownish; about the boundary surface of both the layers there was suspended a brown melanine-like substance. On fractionation of every part the aqueous solution was rejected and the melanine-like substance was dried to give 0.23 g. (4.6% of the original substance). The phosphorus and saccharide reactions of the substance were negative. The ethereal solution was dehydrated with anhydrous sulphate of soda and the ether being removed, 2.40 g. of a mixture of fatty acids were obtained (48% of the original substance). Its iodine value indicated 125 and its neutralisation value 215.

To 2.0365 g. of a mixture of fatty acids 30 c.c. of absolute alcohol and 3.0 g. of lead acetate were added. The mixture was boiled and after being allowed to stand overnight in a cool place it was filtered.

Saturated acids. The precipitate of the lead salt was suspended in water and hydrolysed with hydrochloric acid whereupon solid fatty acids weighing 1.0132 g. (50% of the mixture of fatty acids) were separated. These acids were dissolved in absolute alcohol and crystals of melting-points of 61–63°, 57–58°, 54–57° and 52° were obtained by fractional recrystallisation.

The crystals first fractionated were largest in quantity and on recrystallisation their melting-points indicated 62–62.5° and their neutralisation value was 220. When the recrystallised fatty acid was melted together with palmitic acid its melting-point did not fall,

and the other crystals hardly attained constant melting-points although they were recrystallised over and again. But they could be roughly classified into two sorts, one melting near 62° and the other melting at as low a degree as $53-54^{\circ}$. The neutralisation values of the latter were 240-245. Perhaps myristic acid may have been contained in the latter.

Unsaturated acid. The alcohol was removed from the filtrate from which the precipitate of the lead salt had been removed and the filtrate was hydrolysed with hydrochloric acid, shaken up with an addition of ether and the ethereal layer was separated. On dehydration with anhydrous sulphate of soda the ethereal solution was decolorised with animal charcoal and the ether being removed, 0.7678 g. liquid fatty acids (37.70% of the mixture of fatty acids) were obtained. They smelled fishy and their iodine value was determined by WIJS' method as 184, 235.32 mg. of iodine being absorbed by 0.1280 g. of the sample. During the determination a white precipitate was recognized to appear in WIJS' reagent. The index of refraction was $n_D^{20} = 1.4731$.

Addition of bromine. 0.5700 g. of the liquid acids was dissolved in ether and bromine was added by the ordinary method and a small quantity of a white bromide which resulted was filtered off. The yield was 0.027 g. The excessive bromine was removed from the ethereal solution with thiosulphate of soda; on washing and dehydration the ether was removed and the remainder was treated with petroleum ether to obtain 0.0640 g. of a yellowish insoluble substance (b). When the ether was removed from the petroleum ether solution, on dehydration there remained 0.6240 g. of a liquid yellowish bromide (a).

When the above-mentioned insoluble bromide was treated with hot benzol, 0.0065 g. of an insoluble substance (b) was obtained and on removal of benzol from the solution there remained 0.003 g. of a white bromide (c). To summarise the above is as follows:

Addition of bromine to the liquid acids (0.5700 g.)	Soluble in ether	{ Soluble in petroleum	
		ether 0.6240 g. (a)	
	Insoluble in ether	{ Insoluble in petroleum	
		ether 0.0640 g. (b)	
		{ Soluble in hot benzol	0.0030 g. (c)
		Insoluble in hot benzol	0.0065 g. (d)

(a) was placed in glacial acetic acid; to the mixture was added zinc dust followed by debromination and fatty acid was separated. Its yield was 0.3085 g., its neutralisation value 220, its iodine value 105 and it corresponded to $C_{16}H_{30}O_2$.

(b) was thrown away by mistake, so the melting-points of (c) and (d) were examined. They were as follows:

(c) turned brown at 183° , black at 210° and melted remaining black at 220° .

(d) turned brown at 225° , dark at 235° and turning black melted at 240° .

Detection of the glycerophosphoric acid fraction. On removal of the excessive baryta by passing CO_2 through the above-mentioned filtrate (B) the filtrate was concentrated to about 100 c.c. and precipitated with an addition of alcohol. The precipitate was separated and dissolved in a small quantity of water. The solution was precipitated with an addition of alcohol. By drying the precipitate a pale brown, light earthy powder which weighed 0.650 g. was obtained. The result of the estimation of barium was as follows:

Sample 0.1700 g., $BaCO_4=0.1043$ g., $Ba=36.10\%$ (in glycerophoric acid barium... $Ba=44.67\%$).

Hydrolysis II

To 7.4610 g. of the sample 300 c.c. of 24% baryta water were added to hydrolyse it on the water-bath for 3 hours. On cooling the solution was divided by filtration into residue (A) and filtrate (B).

Detection of fatty acids. The residue (A) was suspended in water and the mixture was shaken up with additions of hydrochloric acid and ether. As before, the aqueous layer became pale yellow and transparent while the ethereal layer was stained brownish, and about the boundary surface of both the layers there was suspended a brown melanine-like substance. On fractionating every part the aqueous layer was stained brownish, and about the boundary surface of both the layers there was suspended a brown melanine-like substance. On fractionating every part the aqueous layer was rejected and the melanine-like substance was dried to a light and brown powder weighing 0.401 g. (5.3 % of the original substance). The phosphorus and saccharide reactions of the powder were negative. On dehydration of the ethereal solution with anhydrous sulphate of

soda the ether was removed and 3.7729 g. of a mixture of fatty acids (50.5% of the original substance) were obtained. After addition of 30 c.c. of absolute alcohol and 3.0 g. of lead acetate the mixture was boiled, allowed to stand overnight in a cool place and filtered.

Saturated acid. The precipitate of the lead salt was suspended in water and hydrolysed with hydrochloric acid. The mixture was shaken up with an addition of ether to yield solid fatty acids weighing 2.17 g. (57% of the mixture of fatty acids). The acids were dissolved in ether and the solution was decolorised with bone black. Then the ether was removed and a fractional crystallisation was carried out from anhydrous methanol to yield crystals which melted at 62°, 58°, 57°–59° and 57°. By repetition of recrystallisation from anhydrous methanol the crystals which first separated out attained a constant melting-point of 62–62.5°. Their neutralisation value was 219 and their melting-point did not change when melted together with palmitic acid. The other crystals hardly attained constant melting-points notwithstanding repeated recrystallisations, but they could be roughly classified into 2 groups, one which melted at about 62°, the other which melted at about 53–54°. The latter's neutralisation value was about 240, and it was probably myristic acid. The residue of the mother liquor of the crystallisation was pale yellow; it melted at 50° and its neutralisation value was 235.

Unsaturated acid. The alcohol was removed from the filtrate which was separated from the precipitate of the lead salt. The filtrate was hydrolysed with hydrochloric acid, extracted with ether, dehydrated and decolorised and on removal of the ether 1.19 g. of a liquid acid (32%) were obtained.

Hydrogenation. All the unsaturated acid was dissolved in absolute alcohol and a hydrogenation using platinum black as catalyst and a fractional recrystallisation from absolute alcohol were carried out as before. First at 66.5° there separated out 0.22 g. of a fatty acid which indicated almost a constant melting-point and had its neutralisation value at 194. When an amide was prepared its melting-point was 95–102. When the acid was melted together with stearic acid prepared from oleic acid its melting-point rose a little, and when it was melted together with palmitic acid its melting-point evidently fell. Therefore the above-mentioned fatty acid probably was not a new acid, but a mixture of crystals of stearic and palmitic acids. In the light of its neutralisation value and the mixed melting-

point table shown by HEHNER and MITCHELL, it was thought to be composed, roughly speaking, of 90% stearic acid and 10% palmitic acid. The other crystals were mostly composed of palmitic acid which melted at 62–62.5° and had a neutralisation value of 220.

Detection of the glycerophosphoric acid fraction. On removal of the excessive baryta by passing CO₂ through the filtrate (B) which was separated from the barium salt of fatty acids after hydrolysis the filtrate (B) was concentrated and precipitated with an addition of alcohol (5–6 volumes) and the precipitate (a) was separated by filtration. The filtrate was again concentrated and precipitated with an addition of alcohol (20 volumes) and precipitate (b) was separated by filtration. Further the filtrate was concentrated to remove the alcohol and shaken up with ether, when a pretty pale green colour appeared. On dehydration the ether was removed and 0.205 g. of an oily substance (c) which gave off a peculiar smell was obtained. After concentration of the aqueous layer by shaking up with ether its phosphorous reaction was tested and found negative, but a fair amount of N was seen in the aqueous layer.

When the oily substance was allowed to stand, there were formed a slight quantity of colourless rhomboidal crystals (m.p. 114–116°). As their quantity was too trivial they could not be purified. Although their phosphorous reaction was negative, it seems they were the combination of a fatty acid and a nitrogenous substance.

When the above-mentioned barium salts were dried in a vacuum desiccator precipitate (a) weighed 0.77 g. and (b) 0.26 g. Precipitate (a) was a pale brown earthy powder and (b) a white powder. The results of the estimations of barium were as follows:

(a) Sample 0.1336 g., BaSO₄=0.0873 g., Ba=38.4%

(b) Sample 0.1084 g., BaSO₄=0.0442 g., Ba=24.0% (in glycerophosphoric acid barium...Ba=44.67%).

Generalisation and considerations.

When the properties of the separated lipid were examined, it was found to consist of two substances, one soluble in ether, petroleum ether and alcohol (small in quantity), the other soluble in ether and petroleum ether and insoluble in alcohol (large in quantity). They produced precipitates when they were treated with excess of acetone and their phosphorous reactions were positive. In general they had

properties similar to those of lecithine and cephaline. Of the two substances, mainly the one of cephaline which was insoluble in alcohol was examined for its properties. It was very hygroscopic, became colloidal when dissolved in water, was unstable to air and light and its melting-point was about 175–180°. Its phosphorous reaction was remarkable, the percentage of the amount of phosphorous contained being 2.84%. It contained the nitrogen and the nitrogen being all in amino form, N: P was 1: 1 and it belonged to the so-called monoaminomonophosphatides.

But compared with other phosphatides it contained a less amount of N and P and its saccharide reaction was slight. Further it contained small quantities of brown pigment and iodine and about 50% fatty acids as main constituents.

When the properties of the fatty acids were examined, they were found to consist of about equal quantities of saturated acids and unsaturated acids. The saturated acids consisted mainly of palmitic acid and a small quantity of myristic acid. The constitution of the unsaturated acids was rather complicated.

First, when the iodine values of the unsaturated acids were estimated using WIJS' reagent a white precipitate was formed. As to this phenomenon many workers⁽³²⁾⁽³⁵⁾ have already made researches and up to date this reaction has been considered as peculiar to fish oils. It is now established that the precipitate was an iodine chloride of some higher unsaturated acid. In the light of this point the possibility of the existence of some higher unsaturated acid in the precipitate is recognized. Then the properties of the unsaturated fatty acids were examined by means of bromination. Liquid bromine soluble in ether and petroleum ether was the principal product and on debromination its neutralisation and iodine values were estimated showing 220 and 105 respectively. Consequently it corresponded to $C_{16}H_{30}O_2$. Also a small quantity of a solid bromide which was soluble in ether and insoluble in petroleum ether was obtained, but unhappily it being thrown away its properties were not fully studied. Moreover, trivial quantities of solid bromides insoluble in ether were obtained. One was soluble in hot benzol and melted at 220° while the other was insoluble in hot benzol and melted at 240°. As their yield was trivial, their properties could not be investigated completely, but from the view-point of the preceding chapter it is thought they were hexa- and octabromides of $C_{16}H_{30}O_2$ and $C_{18}H_{30}O_2$.

Then a hydrogenation of all the unsaturated acids was carried out, the properties of the acids produced were examined, and the existence of a large quantity of palmitic acid and small quantity of stearic acid was recognized. This result coincided quantitatively with the properties of the bromide produced by the bromination mentioned above, and accordingly assured the existence of C_{16} acid. It is not clear whether the original acid which formed the bromide soluble in ether and insoluble in petroleum ether that was discovered in the bromination was C_{16} acid or C_{18} acid.

In short, the unsaturated acids were mostly $C_{18}H_{30}O_2$. The unsaturated acid which has this molecular formula is zoomaric acid which is widely distributed in aquatic animal oils. These facts are of very much interest. As the higher unsaturated acids of C_{18} i.e. $C_{18}H_{28}O_2$ and $C_{18}H_{30}O_2$ are thought probably to occur also in the present material there are some substances in sardine and herring oils which have the same molecular formulae as these. Morocitic acid ($C_{18}H_{28}O_2$) is well known. That these higher unsaturated acids are found to exist in this kind of phosphatide is all the more interesting when the properties of the phosphatide are compared with those of the phosphatide that exists in the internal organs of animals.

What the author wants to state about the glycerophosphoric acid fraction produced by the hydrolysis of phosphatide is that the above-mentioned result arouses a doubt about the amounts of the contained barium. If the precipitates be a glycerophosphoric acid the contained barium must be 44.67% as the molecular formula of the glycerophosphoric acid is $C_3H_7O_6PBa$. However the amounts of the contained barium are all smaller, the compounds containing 36.10% of barium (the precipitate with the addition of 5 volumes of alcohol), 38.4% (the precipitate with the addition of 5 volumes of alcohol) and 24.0% (the precipitate with the addition of 20 volumes of alcohol) being separated, and if every molecule of the compounds contains an atom of barium it must have a much larger molecular weight than a glycerophosphoric acid.

On the hydrolysis of cephaline there is not much literature. According to Leven and Rolf⁽¹⁵⁾ 90% glycerophosphoric acid was separated from lecithine and 20% of the same was separated from cephaline when lecithine or cephaline was shaken for 16 hours with saturated baryta water. They believed it was because the combination of a nitrogenous substance was very strong and consequently it

was very hard to prepare glycerophosphoric acid from cephaline. PARNAS⁽¹⁹⁾ stated that a tetra basic acid which contained phosphorus but not nitrogen was produced when cephaline was hydrolysed with baryta water. KOGANEI⁽¹⁰⁾ reported that a compound of cholamine and a fatty was produced when cephaline was hydrolysed with an acid and that this compound was soluble in ether and very stable to mineral acids.

In the light of these points it seems to be a peculiarity of the hydrolysis of cephaline that it is not so easy as that of lecithine and it is not improbable that the substances separated from the glycerophosphoric acid fractions in the author's test might not necessarily coincide with the glycerophosphoric acid, but this point necessitates further study for elucidation. Cephaline is distributed widely in the animal fibrin, especially largely in brain, and in nerve and kidney also. When the properties of these cephalines are examined it is seen that every fatty acid contained in them contains one mol of saturated and unsaturated acids respectively, that the saturated acid is mostly stearic acid and that the unsaturated acid is oleic, linolic, cephalinic, arachidonic or various other acids (cephalinic acid is an isomer of linolic acid). But the composition of fatty acids contained in the cephalin-compound separated in the author's test is quite different. Although, as above-stated, the separated cephaline-like phosphatide belongs to the monoaminomonophosphatides, it contains less N and P compared with other cephalines and its saccharide reaction is slightly positive. It contains traces of brown pigment and iodine, and as to the fatty acids, its chief constituents, there are several kinds both saturated and unsaturated. When all these points are put together it is thought that the phosphatide may not be a simple cephaline, but may be a mixture of cephalines, and these cephalines may form the so-called higher phosphatide which is in combination with various substances.

C. Summary

A sort of phosphatide was separated from the blades of *Alaria crassifolia* KJELLM. This was found to be mainly constituted of cephaline that belongs to the monoaminomonophosphatides. It was perhaps a mixture of cephalines of this sort. It contained about 50% fatty acids. The fatty acids were constituted in general of equal quantities of saturated and unsaturated acids; the saturated acids

were constituted mainly of palmitic acid and besides contained a small quantity of myristic acid; the unsaturated acids were constituted mainly of $C_{16}H_{30}O_2$ and besides contained an acid which by bromination formed a solid bromide soluble in ether and insoluble in petroleum ether and two sorts of higher unsaturated acids belonging to C_{18} .

V. The Brown Substratum of Phaeophyceae

As to the substance which gives brown colour to *Phaeophyceae* many researches from various standpoints have been made from a fairly long time ago, but its nature is not yet clear and at present it is generally looked upon vaguely as a variation of tannin. At first Cohn⁽¹¹⁾⁽¹⁸⁾ held in 1865 that *phaeophyceae* contained only a kind of brown pigment and he named it phaeophyll. He stated in 1867⁽¹¹⁾⁽¹⁸⁾ that this pigment would perhaps have the same function as the chlorophyll of the higher plants and its combination form might be similar to that of chlorophyll.

Afterwards in 1869 Millardet⁽¹¹⁾ recognized that there was a brown pigment which was water-soluble and named it "Phykophaein". Later, in 1876, Reinke⁽¹¹⁾ recognized "Phykophaein" too, but he said this brown pigment might be produced when seaweeds were dried.

Thereafter on the side of botanists Berthold⁽¹⁸⁾ recognized in 1882 for the first time the existence of a large number of vesicles (blaeschen) that contained tannin in seaweeds of various kinds. Later the existence of the vesicles was confirmed by many botanists. Crato⁽¹⁸⁾ gave the name of physoden to these vesicles. He thought the contents of physoden to be a phloroglucine-like substance as they gave a red coloration by vanillin hydrochloric acid. But Moeller⁽¹⁸⁾ maintained that they were tannin on the ground that the same reaction was seen with tannin. Later on there appeared an opinion that they might be an unsaturated fatty substance as they turned black upon treatment with ammoniacal silver solution or osmic acid. But HUNGAR⁽¹⁸⁾ (1902) denied the opinion of the existence of an unsaturated fat as the substance which give an osmic reaction remained insoluble although the contents of the vesicles were dissolved out by fat solvents such as benzol, chloroform or carbon disulphide.

Later MOLISCH⁽¹⁸⁾ (1905) recognized the existence of phy-

cophaïn too, but he supported the theory that this pigment was formed after the seaweeds were dead. TSWETT⁽¹⁸⁾ (1906) reported that he had made an aqueous extract of seaweeds to observe the pigment and had known its oxidation to proceed turning brown from colourless when it had been alkalisèd.

Thereafter KYLIN⁽¹¹⁾⁽¹²⁾ (1912, 1913) reviewed and reconciled the literature concerning the brown pigment published up to that time by both chemists and botanists and carried out experiments to establish the tannin transformation theory prevalent nowadays. First he ascertained the presence of physoden in 50 kinds of seaweeds and demonstrated that physodens were vesicles of various sizes that moved about restlessly in the protoplasm of a cell. He gave the small physoden a special name "Fucosanblase" and termed their contents "Fucosan". KYLIN stated that small physodens were scattered mainly about the circumference of a cell and large physodens (composed of united vesicles) piled up around the cell nucleus like a cluster of grapes. When he investigated the distribution of physodens, he found that they existed specially in large quantities in the tissues that performed assimilation and in the reproductive organs. In the course of his researches about the nature of fucosan he recognized it had a strong property of reducing silver, iron and copper salts and was precipitated by neutral lead acetate and that its acid solution was astringent as tannin solution. He looked upon fucosan as similar to tannin though it was not typical tannin. Further KYLIN recognized that the aqueous solution of fucosan was oxidised specially fast when it gave the ammoniacal alkaline reaction; it was oxidised slowly in ordinary temperature and fast in high temperature when it was neutral and it was oxidised very slowly when it was acid. He further stated that the changes of colour by oxidation occurred in the order yellow→yellowish-brown→brown→dark reddish-brown and the last transformed substance corresponded to phycophaine. He held that the phycophaine was probably produced by oxidation after the seaweeds were dead.

Thereafter hardly any one reported about the brown pigment until 1931 when TAKEO TAKAHASHI extracted with cold alcohol the dried powder of *Ecklonia cava* which belonged to *Laminaria* and separated what was soluble in ether. He carried out a saponification and separated a dark brown pigment that was insoluble in ether (it was dissolved in one normal NaOH solution and precipitated by

immediate acidification with sulphuric acid). According to TAKAHASHI this pigment did not contain sulphur, phosphorus, arsenic and halogens as examined by VOLHARD's method; it did not contain nitrogen as it did not produce a cyanide when it was fused with metallic sodium; it produced a substance which gave the phloroglucin reaction when it was fused with potassium; further it had phenol character, a reducing property and unsaturated nature and gave no saccharide reaction. TAKAHASHI supposed the chemical formula $C_{46}H_{38}O_8(OH)_{10}(CO)_2$ for the pigment and stated that it seemed to be very similar to phlobaphene, a brown pigment contained in the bark of higher plants and produced from degenerated tannin. However, he stated nothing about the combination form of the original of this pigment at all. As stated above, there have been many researches on the brown substratum of *Phaeophyceae* hithertofore, but one can not help thinking that we have only a scanty and vague knowledge of its combination form as yet.

A. Isolations and combination forms of the brown substance containing phosphorus

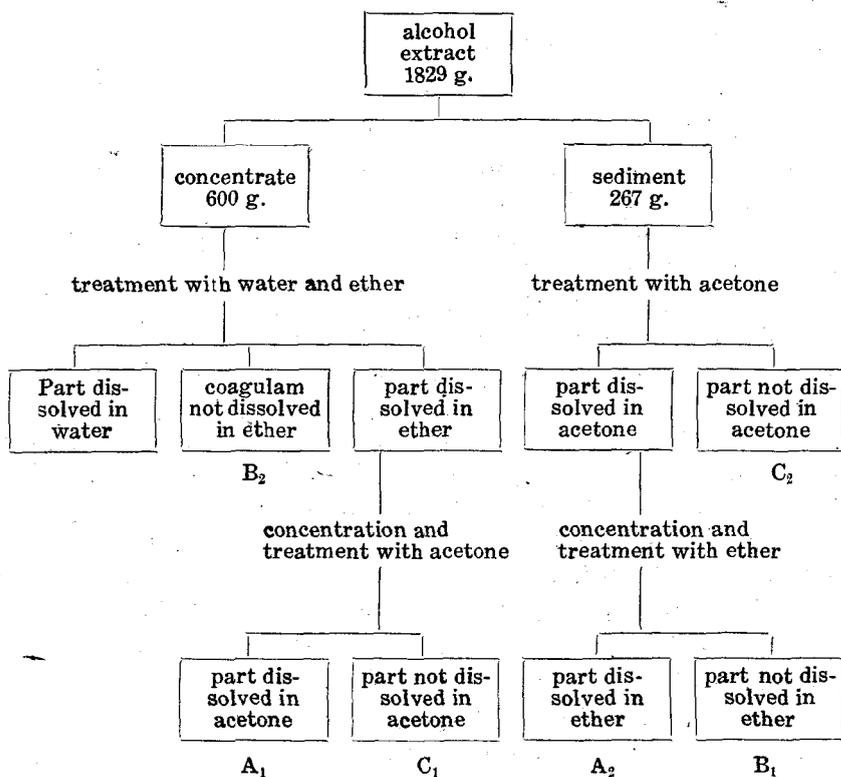
As the brown substratum of *Phaeophyceae* was easily soluble in alcohol the author proposed to separate it by means of this solvent and the idea being practised on *Cystophyllum hakodatense* YENDO various brown substances containing phosphorus were obtained and their combination forms were examined.

Twenty-seven kg. of the dried powder of *Cystophyllum hakodatense* YENDO were taken and digested in 3 kg. lots with 4 l. of 98% alcohol for 3-4 hours and the extract was filtered. Two liter of alcohol were added to the residue to carry out a digestion for 3 hours and the extract was filtered as before. The two filtrate were added together and allowed to cool, on removal of the crystals of mannit and other impurities the mixture was concentrated into a black tarry syrupy liquid (yield 1829 g.). When the syrup was put aside for a long time a thick sediment (267 g.) accumulated on the bottom of the vessel and the upper part became a rather easily flowing liquid, so by decantation the two parts were separated and the liquid part was concentrated to a thick liquid of 600 g.

When to the concentrated syrup liquid was added first a little quantity of water then a large quantity of ether and shaken up, the

aqueous solution turned yellowish-brown (it contained water-soluble yellowish-brown pigment and was used for the detection of bases). The ethereal solution assumed a thick dark brown colour and a very hydrophile black coagulum (B_2) occurred near the boundary surface of the two solutions. The ethereal solutions were concentrated and on removal of the ether the concentrate was poured with stirring into a large quantity of acetone whereupon a dark brown insoluble substance (C_1) was precipitated. The precipitate was filtered off, the filtrate was concentrated and the acetone being expelled the filtrate turned into a dark brown oil (A_1).

Next the sediment was dissolved directly in acetone and on removal of the insoluble substance (C_2) the solution was concentrated and poured with stirring into a large quantity of ether and there occurred a dark brown coagulum (B_1) (as B_2 this substance swelled easily and formed a hydrosol when it came in contact with water and it coagulated when it was treated with ether). The coagulum was



separated and the ethereal solution was concentrated into a dark brown oil (A_2), like (A_1). The above-described operations are shown in the next scheme.

The products obtained by the above-described operations were mixed together as the following equations and the mixtures were treated as described next below.

$$A_1 + A_2 = a, \quad B_1 + B_2 = b, \quad C_1 + C_2 = c$$

Treatment of a. An extraction was carried out with an addition of 80% methanol little by little and thorough working with a glass rod. All the residue was dissolved in petroleum ether and the solution was shaken up with an addition of 90% methanol little by little to be fractionated. Finally a was separated into three parts as follows:

- (1) part dissolved in 80% methanol (yellowish-brown)..... yields of concentrated 17 g.
- (2) part dissolved in 90% methanol (brownish-yellow) .. (15 g.)
- (3) part dissolved in petroleum ether (brown).....368 g.

As treatment (1) has already been described in Chapter III it will not be touched upon now. (2) was allowed to stand as a solution for about 2 weeks and as a black tarry sediment was formed it was separated by decantation. When dried and pulverised it turned a brown powder of which the yield was 10 g. (hereafter the powder will be called γ). The part dissolved in alcohol was concentrated and saponified through which a small quantity of unsaponifiable matter containing vitamin and 3.4 g. of a mixture of fatty acids was produced.

Next, the oil of (3) was saponified with 500 c.c. of 10% alcoholic solution of NaOH on a water-bath for an hour (during which an odour of amine was emitted strikingly and red litmus was turned blue) and on removal of the excessive alcohol the mixture was shaken up with additions first of a large quantity of water and then of ether. On separation of unsaponifiable matter the aqueous solution was acidified with sulphuric acid and shaken up with an addition of ether. Then the ethereal solution assumed a brown colour and dissolved out a large quantity of fatty acids, the acid aqueous solution turned blue and transparent and a dark brown coagulum occurred near the boundary surface of both the solutions. The coagulum was dissolved in an alkaline solution which was immediately acidified

with sulphuric acid (the solution would turn black if it was allowed to remain alkaline for a long time), whereupon a voluminous dark brown precipitate occurred. The precipitate was coagulated with an addition of a large quantity of ether and the coagulum was dissolved in a small quantity of a dilute alkaline solution and the same treatment as above was carried out. The procedure was repeated until the added ether was not stained brown and the coagulum was separated, dried in a desiccator *in vacuo* over sulphuric acid and pulverised. It was a dark brown powder and weighed 35 g. (hereafter the substance obtained by the same procedure will be termed a brown pigment and the original compound of the brown pigment before saponification will be called α).

Treatment of b. The whole substance was dissolved again in a small quantity of acetone and precipitated by being poured with stirring into a large quantity of ether; the precipitate was dissolved in a small quantity of acetone and the same treatment as above was carried out. The procedure was repeated until hardly any more acetone dissolved out and the precipitate was separated. When the precipitate was dried in a desiccator *in vacuo* over sulphuric acid it became a light block-like solid which was bright black resembling a fragment of anthracite and when the solid was pulverised it broke easily to form a brown powder of which the yield was 16 g. For further purification the powder was infused with warm water at 40°C. again and again until water dissolved out nothing more at all and dried (it lost its hydrophile property completely and its infusion with water became exceedingly easy, unlike before the treatment with warm water). After drying the powder became almost insoluble in acetone and when it was treated with acetone to remove the soluble part, washed in ether and dried the yield was almost unchanged being about 16 g. which contained 10.26% water (this will be called α).

Treatment of c. c was dissolved in a small quantity of ether and was precipitated by being poured with stirring into a large quantity of acetone; the precipitate was dissolved again in a small quantity of ether and treated as above; the precipitate was taken out and dried in a desiccator *in vacuo* over sulphuric acid. It was a dark brown solid resinous matter amounting to 6 g. (it will be called β).

Chemical Properties

Properties of a. When fresh, this material was easily soluble in alcohol and acetone and insoluble in ether. It was very strong in its hydrophile property and had a strong inclination to form a hydrosol, but this property weakened gradually and after the treatment with warm water it became to dissolve with difficulty in alcohol, acetone and dilute alkaline solutions and its hydrophile property was lost completely. Its chemical properties were ascertained as follows:

(Phosphorus). To a small quantity of the sample baryta water was added and on evaporation to dryness it was burned. The residue was dissolved in nitric acid and the solution was treated with ammonium molybdate solution, whereupon a yellow precipitate occurred manifestly. So the estimation of a phosphate was carried out. One g. of the sample was taken and burned as above, the residue was dissolved in hydrochloric acid, Ba was removed by adding sulphuric acid, the solution was alkalisied with ammonia, a magnesia mixture was added to produce a precipitate and on estimation by the ordinary method 8.6 mg. of $Mg_2P_2O_7$ were obtained. The amount of P was 0.224%. The amount of P in proportion to that of the anhydride of the sample was 0.247%.

(Nitrogen). When an alkaline solution was added to a small quantity of the sample and the mixture was heated, the mixture easily evolved an odour of amine, turned red litmus blue and further formed manifestly a cyanide with metallic sodium. So nitrogen was estimated by KJELDAHL's method and found to be 1.25%. Its amount in proportion to that of the anhydride of the sample was 1.39%.

(Reducing property). To 0.0200 g. of the sample was added FEHLING's solution and treated as in the case of the estimation of saccharides. The yield was 17.3 mg. of Cu_2O . The ratio of its amount to the amount of the sample was 0.865 g. to 1 g. and the ratio of its amount to that of the anhydride of the sample was 0.953 g. to 1 g. (hereafter the number of the grams of Cu_2O which is proportionate to 1 g. of the anhydride of the sample will be termed the reduction value).

(Hydrolysis I). Fifty c.c. of 3% baryta water was added to 1 g. of the sample and a hydrolysis was carried out on a boiling water-bath for 5 hours. Immediately after the commencement of the hydrolysis an odour of amine was evolved and the solution turned

red litmus blue. After hydrolysis the solution turned slightly brown, by the most part of the sample remained as a dark brown residue.

Residue. The residue was suspended in water, the mixture was shaken up with an addition of hydrochloric acid and ether and the ethereal solution was separated. After with water and dehydration, the ether was removed from the solution leaving hardly any residue. The part of the residue insoluble in ether was dissolved in a dilute alkaline solution after washing with water and when the solution was slightly acidified there appeared a precipitate. The precipitate was separated and dissolved in alcohol, and filtered. The filtrate was precipitated with addition of excess of ether and the precipitate being dried became a dark brown lump which was pulverised into a fine powder. When the phosphorous reaction of the powder was tested, it was found almost negative and when the powder was treated with metallic sodium it formed a manifest cyanide. When it was treated with FEHLING'S solution there was produced a red precipitate of Cu_2O . A small quantity of the sample was distilled with zinc dust. The distillate obtained dyed red a piece of pine wood wet with hydrochloric acid and produced a deep blue precipitate when shaken up with an addition of isatin and dilute sulphuric acid. The precipitate dissolved in glacial acetic acid and concentrated sulphuric acid. The distillate mentioned above turned violet in the aqueous solution of quinone and its pigment passed into ether. Five c.c. of the aqueous solution of the distillate turned yellowish-green when added 0.2 c.c. of 5% nitroprusside solution and 1 c.c. of NaOH solution, gradually passed to green and turned blue when heated with an addition of glacial acetic acid. Therefore it was known that there were pyrrole nuclei in the sample.

To small quantity of the sample was added 50% KOH solution and evaporated to dryness on a water-bath. The solid obtained was dissolved in water and the solution was filtered. When the filtrate was acidified with sulphuric acid, there evolved a peculiar odour along with CO_2 . On filtration the solution was shaken up with an addition of ether, and on removal of the ether, the remainder was dissolved in hot water. Neutral lead acetate was then added and filtered. The lead was removed with an addition of sulphuric acid and the solution was shaken up with ether. After removing the ether the remainder was dissolved in hot water again. When this solution was tested, it turned pine wood wetted with concentrated hydrochloric

acid red and turned the ferric chloride solution reddish violet. Therefore it was recognized that phloroglucine was produced.

Filtrate. By passing CO_2 through the filtrate obtained by the above described hydrolysis Ba was removed; when about 3 volumes of alcohol were added, a white colloidal precipitate occurred. When the precipitate was hydrolysed with concentrated nitric acid and ammonium molybdate solution added there formed a yellow precipitate which showed that the phosphorous reaction of the solution was positive.

(Hydrolysis II). To five grams of the sample were added 100 c.c. of water and 5 g. of caustic baryta water. Hydrolysis was carried out on a water-bath for 3 hours, and the solution was shaken up with an addition of ether to remove the soluble substances. The solution was acidified with sulphuric acid and shaken up with an addition of ether. The ethereal solution was separated, washed with water and dehydrated with anhydride of sodium sulphate, and when the ether was removed there was hardly any residue left. Considering from these results it was known that there was hardly any fatty acids in the sample. The same tests of the residue as described above were carried out and quite the same results were obtained. It was ascertained that the residue of saponification, that is, the brown pigment, contained hardly any phosphorus, but did contain nitrogen. Further it gave pyrrol and phloroglucine reactions likewise as described above and possessed a reducing property.

(Hydrolysis III). To 5 g. of the sample were added 100 c.c. of 5% H_2SO_4 , hydrolysis was carried out on a boiling water-bath for 5 hours and the solution was filtered.

Residue. The residue was washed well with water and dried up. It was different in its properties from the residue obtained in a hydrolysis with an alkali and dissolved with difficulty in dilute alkalis and alcohol, but easily in concentrated alkalis. When tested after burning, its phosphorous reaction was slightly positive.

Filtrate. The solution was yellowish-brown. When tested after neutralisation with CaCO_3 its phosphorus reaction was almost negative. It did not reduce FEHLING'S solution. After decolouration with charcoal it was concentrated and boiled with an addition of phenylhydrazine hydrochloride and sodium acetate, but it produced no osazone; therefore it was doubtful whether any saccharides existed in the solution.

(Hydrolysis IV). To the residue of Hydrolysis III was added 100 c.c. of 10% HCl, hydrolysis was carried out on a water-bath for 8 hours and the solution was filtered.

Residue. When the residue was dried up after thorough washing with water it turned a black substance like charcoal containing 11.0% of water. When estimated by KJELDAHL's method the amount of nitrogen contained in it was 1.40% of the anhydride of the sample. When distilled with zinc dust it gave the pyrrole reaction. When boiled in an alkaline solution it did not give off an odour of amine at all and the solution did not turn red litmus blue. When fused with potassium it gave the phloroglucine reaction. Its reduction value was 0.621.

Filtrate. When the filtrate of the hydrolysis was neutralised and alkalisied slightly with NaOH solution there resulted a small quantity of a brown colloidal precipitate. The precipitate was filtered, dried and burned. Phosphorous reaction was tested and found positive. While the filtrate therefrom gave the reaction slightly.

(Hydrolysis V). With an addition of 50 c.c. of 5% H_2SO_4 2 g. of the sample were hydrolysed under pressure in an autoclave at $150^\circ C$. for 3 hours; after more 2 hours the solution was taken out and filtered. The filtrate was brown and a black residue like a charcoal was obtained.

Residue. When well washed with water and dried up the residue turned to a charcoal like powder containing 6.0% water. Five-tenths g. of it was taken and burned for the test of the phosphorus reaction which was found to be negative. When nitrogen was estimated it was found to be 1.48% of the anhydride of the sample. The reduction value was 0.691.

Filtrate. The filtrate was neutralised with $CaCO_3$ and filtered. With lead acetate a brown colloidal precipitate was formed from the filtrate, and after lead was removed by passing H_2S the filtrate was concentrated to 250 c.c. The following tests were executed.

- (1) *Reducing property.* To 20 c.c. of the filtrate FEHLING's solution was added and 6 mg. of Cu_2O was produced. The amount corresponded to 0.0375 g. to 1 g. of the sample. If the reduction value of the sample be presumed 100, it should have corresponded to 4.3%. It could not be thought that

the reducing property of the filtrate was attributable to saccharide.

- (2) *Glycerine reaction.* One hundred c.c. of the filtrate were evaporated until almost dried up, potassium thiosulphate was added the whole was heated whereupon a pungent odour of acrolein was noticed. When a small quantity of the sample was tested for DANSTAN'S reaction, it was positive (when the sample was put in 5% solution of borax which was turned red with drops of phenolphthalein turned colourless and when heated it turned red again).
- (3) *Test for mannit.* The procedure for preparing a soluble copper salt by WAGENAAR'S method was carried out with 50 c.c. of the sample. An attempt was made to titrate the solution with $\text{Na}_2\text{S}_2\text{O}_3$ but the solution did not require $\text{Na}_2\text{S}_2\text{O}_3$ solution at all and being colourless and transparent did not indicate the presence of a soluble copper salt.
- (4) *Test for bases.* The remaining solution was acidified with sulphuric acid and treated with Na phosphotungstate, but no precipitate was produced.

The chemical properties of *a* observed from the above described results will be described as follows. *a* contained 0.24% phosphorus and 1.39% nitrogen. On saponification with an alkali it gave off easily an odour of amine and produced the phosphorous compounds along with a large amount of a dark brown residue, but no fatty acids. One of the phosphorous compounds was a substance like glycerophosphoric acid and another substance was slightly alkaline and positive in the phosphorus reaction and appeared as a brown colloidal precipitate. The presence of saccharides in it was doubtful, but that of glycerine was thought possible. A brown or dark brown matter produced by saponification was soluble in a dilute alkaline solution and precipitated in an acid solution. The precipitate was easily soluble in alcohol and precipitated by ether, but a brown matter produced in the hydrolysis with an acid was different. It was soluble with difficulty in a dilute alkaline solution and alcohol. However, both of them contained nitrogen, liberated pyrrole in a distillation with zinc dust, produced a substance giving the phloroglucine reaction by fusing with potassium and also possessed reductibility. The amount of nitrogen and the reduction value seemed to differ a little

according to the degree of hydrolysis. The precipitate which contained 1.40% nitrogen showed the reduction value of 0.621 and that which contained 1.48% nitrogen the value of 0.691.

In sum it may be concluded that the brown substratum *a* had a constitution just like that of a phosphatide, containing a acidic brown pigment instead of fatty acids. Further it was presumed that it contained amine instead of nonvolatile base.

Properties of β . β was not reduced to powder as *a*, but appeared like a dark brown fat. While fresh, it was hydrophile and formed a hydrosol absorbing water, but it gradually became insoluble. As the yield was small, its properties were not sufficiently investigated, but some chemical properties may be shown as follows:

(Phosphorus). When 1.1277 g. of the sample was treated as in the case of *a*, 10.0 mg. of $Mg_2P_2O_7$ were obtained and that meant 0.25% for P.

(Nitrogen). Nitrogen was 1.5% as determined by KJELDAHL's method. When β was boiled in an alkaline solution an odour of amine was emitted and the solution turned red litmus blue.

(Reducing property). The reduction value was about 0.200. The estimation was very hard, owing to containing a small quantity of fat, producing from vigorously when boiled with FEHLING's solution.

Though the sample was taken so small amount as 0.02 g. it was hard to carry out the estimation. The results obtained, was therefore hardly deemed to be exact.

(Hydrolysis). Fifty c.c. of water and 2.5 g. of baryta water were added to 3.99 g. of the sample, and hydrolysis was carried out on a water-bath for 3 hours. On cooling, the solution was filtered.

Residue. The residue was suspended in water and the mixture was acidified with hydrochloric acid and shaken up with an addition of ether. The ethereal solution which was stained brown was separated, washed with water, dehydrated and decolourized. When the ether was removed from the solution about 0.1 g. of yellowish-brown fatty acids was obtained (as decoloration was carried out the actual yield was uncertain). The iodine value was 83 and WIJS' indicator did not become turbid white in the determination.

The black substance which was coagulated when the solution was shaken up with an addition of ether was dissolved in alcohol, precipitated by ether and dried brown. Its phosphorus reaction was

slightly positive, it had a reducing property, formed a cyanide with metallic sodium and produced pyrrole by distillation with zinc dust. Thus this substance gave quite the same reactions as those which the pigment obtained in the case of α .

Filtrate. To the filtrate obtained by the hydrolysis described above about 3 volumes of alcohol was added, on removal of Ba by passing CO_2 , a white precipitate was produced. The phosphorus reaction of the precipitate was positive.

When considered from these results β had the same properties as α , generally speaking. Only a small quantity of fatty acids was obtained in the case of β . However, it was uncertain whether they were constituents of β or not for the amount of them was not estimated.

Properties of γ . γ was the substance precipitated as a sediment from the 90% methanol extract of the parts soluble in acetone, ether and petroleum ether and might be thought as a part of δ which will be described next. When it was dried up into a powder it assumed a brown colour and dissolved easily in alcohol. The alcoholic solution gave a brown colour and a slight red phosphorescence. When the spectrum of the alcoholic solution was examined, a wide absorption band in the red region, a weak and slender band near the yellow region, a weak and broad band in the green region and a broad band in the blue region were seen. It gave evidently the phosphate reaction like α and β ; when it was heated in an alkaline solution an odour of amine was evolved and the solution turned red litmus blue. Its chemical properties will be described briefly as follows:

(Phosphorus). 12.7 mg. of $\text{Mg}_2\text{P}_2\text{O}_7$ were obtained from every 1 g. of the sample. It meant 0.25% of P. P occupied 0.27% in proportion to the anhydride of the sample.

(Nitrogen). When estimated by KJELDAHL'S method, nitrogen was 1.01%. It amounted to 1.08% in proportion to the anhydride of the sample.

(Reducing property). When the reduction value was determined it was 0.295.

(Hydrolysis I). Fifty c.c. of water and 2.5 g. of baryta water were added to 2 g. of the sample and hydrolysis was carried out on a water-bath for 3 hours. On cooling, the solution was filtered and the residue was separated.

Residue. The residue was suspended in water and the solution was acidified with hydrochloric acid. When the solution was shaken with an addition of ether a black coagulum appeared near the boundary surface of both solutions. So on fractionation into the following 3 parts the chemical properties of them were examined.

- (a) the party dissolved in ether (brownish-yellow).
- (b) the black coagulum.
- (c) the liquid part acidified with hydrochloric acid.

Treatment of (a). After removal of the acid by washing with water again and again, (a) was dehydrated with anhydride of sodium sulphate and on removal of ether from it 0.387 g. of fatty acids (19.35% in ratio to the original) were obtained. When the iodine value was determined again after dehydration it was 171 and the indicator used produced white turbidity as before.

Treatment of (b). The coagulum (b) was dissolved in a dilute alkaline solution, the insoluble substance was removed, and when the solution was acidified with hydrochloric acid a dark brown precipitate was produced. The precipitate was coagulated with an addition of ether, dried and pulverised. The yield was 0.5 g. It was easily soluble in alcohol and when concentrated hydrochloric acid, concentrated sulphuric acid or concentrated nitric acid was added to it, a yellowish-brown coloration always resulted.

The reduction value of the pigment was determined to be 0.70. The phosphorus reaction was slight. After the pigment was precipitated from the alcoholic solution by pouring into a large quantity of ether and again dissolved in a small quantity of alcohol and treated as before for purification the properties of the pigment were examined. The reduction value was almost the same being 0.70. When nitrogen was estimated by KJELDAHL's method, it was 0.906%. The pyrrole and phloroglucine reactions were evident too.

Treatment of (c). When (c) was neutralised and alkalisied slightly with NaOH solution, a dark brown colloidal precipitate was produced. The precipitate was separated and dried to the yield of about 0.1 g. It gave the phosphorus reaction slightly and resembled closely in appearance the product described in the paragraph on the hydrolysis of *a*. When the precipitate was filtered off and the filtrate was concentrated continuing its alkalinity, a white colloidal precipitate was produced. Though this precipitate disappeared when it was

acidified, appeared again when it was alkalisied and gave a striking phosphorus reaction.

Treatment of the filtrate. When Ba was removed from the filtrate of the hydrolysis mentioned above by passing CO_2 gas and to the filtrate were added about 3 volumes of alcohol, a white colloidal precipitate was produced. When this precipitate was separated and dried, the yield was about 0.12 g. Its phosphate reaction was positive and in appearance it seemed to be glycerophosphoric acid. When the filtrate from which this precipitate was separated was decolorised with charcoal and concentrated, it turned a viscous colourless solution. When a part of the solution was taken and heated with an addition of potassium thiosulphate, a stimulating odour peculiar to acrolein was evolved. Perhaps glycerine was present.

(Hydrolysis II). Fifty c.c. of 5% H_2SO_4 was added to 1 g. of the sample and hydrolysis was made on a water-bath for 4 hours. The solution was filtered and the residue was washed well with water and dried up into a dark brown powder amounting to 0.7 g.

Residue. Thus dark brown pigment was different from the above-mentioned pigment obtained in the hydrolysis with an alkaline solution. It dissolved with difficulty in alcohol and dilute alkaline solution; concentrated hydrochloric acid, concentrated sulphuric acid and concentrated nitric acid hardly reacted to the alcoholic solution of this pigment giving scarcely any peculiar coloration. When the reduction value was determined of the pigment washed with alcohol and dried up, it was 0.75. When nitrogen was estimated, its amount was 1.40%. The phosphorus reaction was positive when tested. Further, when the pigment was distilled with zinc dust, pyrrole was produced and when it was fused with potassium a substance which gave the phloroglucine reaction was produced.

Filtrate. When the phosphorus reaction was tested or a part of the filtrate from which the residue was removed, it was negative. When the filtrate was treated with phosphotungstic acid, a little quantity of precipitate was produced. This precipitate was separated by filtering off and hydrolysed with baryta. The solution was passed with CO_2 for removal of Ba, concentrated and added phosphotungstic acid again, when it seemed to turn somewhat turbid (the presence of a base was doubtful).

(Hydrolysis III). Fifty c.c. of 1% H_2SO_4 was added to 1 g. of the sample and a hydrolysis was carried by boiling the mixture for

1 hour. The solution was filtered and the filtrate was neutralised with CaCO_3 , decolorised and concentrated. When FEHLING's solution was added to a part of the concentrate and heated, no precipitate of cuprous oxide was produced and when phenylhydrazine hydrochloride with sodium acetate was added to another part of the concentrate and heated, no osazone was produced. Consequently no presence of a saccharid was to be recognized.

When observed in the light of the above-described results γ contained 0.27% phosphorus and 1.08% of nitrogen, emitted easily an odour of amine in the saponification with an alkali. Further it produced a large quantity of the brown pigment along with fatty acid, glycerine, a glycerophosphoric acid like substance, bases (?) and some phosphorous compound. But did not produce saccharides in the hydrolysis with an acid and had a constitution similiar to β . The brown pigment gave the pyrrole and phloroglucine reaction as in the other cases. Some brown pigment contained 0.91% of nitrogen while its reduction value was 0.70. Its hydrolysis with an acid was more difficult than that with an alkali and its phosphate reaction seemed to be stronger than the residue of the hydrolysis with an alkali.

Although the amount of fatty acids produced in this case was larger than that of β , the iodine value of γ indicated 171 and γ gave the higher unsaturated acid reaction. Fatty acids could not considered as constituents of γ when observed from the view-point of the purity of it, but it was doubtful that a part of fat which had coexisted before the separation had passed into γ . As the author has reported elsewhere on the properties of the fat which coexisted with γ , it contained 37.60% of higher unsaturated acids in ratio to the total free fatty acids.

Properties of δ . δ was a substance to be studied as the mother substance of γ . It easily dissolved in acetone, ether and petroleum ether, but not in 90% methanol and could not be separated. However, observed from the facts that in its saponification an odour of amine was easily evolved and a trace of phosphorus was left in the brown pigment, and from the properties of the latter, it could be supposed that δ had a similar constitution to the other substances. The description of the brown pigment obtained from δ will be omitted here and will be given somewhat circumstancially in the next chapter.

Volatile bases. When the brown phosphorous compounds such as the above-mentioned α , β , γ and δ were treated with dilute alkalis, they easily evolved an odour of amine and turned red litmus paper blue. When the presence of nonvolatile bases in the hydrolysed solution was investigated, they were hardly recognized there. From the viewpoint of these facts amine combinations in the phosphorous compound could be guessed. So to examine the properties of the volatile amines of this kind first, 3% baryta water was added to a small quantity of the sample and it was distilled by boiling. The distillate was introduced into dilute hydrochloric acid and evaporated to dryness to produce a small quantity of crystals. The crystals were separated, dried in a desiccator *in vacuo* over sulphuric acid and treated with cold absolute alcohol to remove the insoluble ammonium chloride. The part dissolved in alcohol underwent the expulsion of alcohol, decoloration with bone black, drying again in a desiccator *in vacuo* and was next treated with chloroform. The part dissolved in chloroform was separated and when the chloroform was removed a small quantity of hygroscopic, colourless, needle-like crystal was obtained. When these crystals were heated in an alkaline solution a peculiar odour of amine was evolved. The solution was precipitated with potassium iodide containing a small quantity of hydrochloric acid and a brown precipitate was formed by the treatment of gold chloride solution containing sodium bromide. When picric acid salt was prepared as violet needle-like crystals whose melting-point indicated 213° coinciding with that of trimethylamine.

Summary

A dried powder of *Cystophyllum hakodatense* YENDO was infused in alcohol and by the treatment of the infusion with acetone, ether, petroleum ether and 90% methanol 4 kinds of brown substrata were separated. These substances contained about 0.25% of phosphorus and 1–1.5% of nitrogen, and gave off easily the odour of amine when saponified with alkalis. On examination it was found that trimethylamine was produced. Further a large quantity of acid brown pigment together with glycerine, fatty acids, several phosphorous compound and nonvolatile bases (?) were liberated in the saponification. They did not produce any saccharide in acid hydrolysis and had just such constitutions as were seen in phosphatides. But they seemed to contain acid brown pigment instead of fatty acids or both

fatty acid and brown pigment and to combine trimethylamine instead of nonvolatile bases. The brown pigments contained about 1.4% of nitrogen and had reducing properties although they were diverse depending on the degrees of their hydrolysis (0.7 g. of Cu_2O were produced by FEHLING's solution in ratio to 1 g. of the anhydride of the sample). They seemed to be the degenerated substances of chlorophyll or tannin as they distilled over pyrrole by distillation with zinc dust and produced a substance which gave the phloroglucine reaction by fusion with potassium. The brown substratum was thought to be a sort of lipoide as a whole.

B. Brown pigment and its physiological function

It was stated in the preceding section that a nitrogenous brown pigment not containing phosphorus, fatty acid and amine had been obtained when a brown phosphorous compound which had passed into alcohol had been saponified. In this section the chemical and biochemical properties of a brown pigment which was obtained from a brown substratum δ will be described.

1) Chemical properties of the brown pigment

General properties. This pigment dissolved easily in a dilute alkali and alcohol giving the solution some viscosity. When the relative viscosity of 0.2% KOH solution of it was examined, the following results were obtained.

Viscosity of the alkaline solution of the brown pigment
(by Ostwald's viscosimeter).

Concentration of pigment (%)	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7
Time of flowing down (seconds)	6.7	6.8	7.0	7.4	7.6	8.0	8.0	8.0
Relative viscosity	8.0/6.7=1.19							

The alcoholic solution gave a brown colour and emitted red phosphorescence. When the spectrum was examined an absorption band was seen in the centre of the red region and a weak but wide one and a narrow one in the green region. The pigment contained 1.27% of ash and phosphorus reaction was slightly. It contained

1.28% of nitrogen and its reduction value indicated 0.80. The results of its elementary analysis were as follows:

Quantities of material (mg.)	CO ₂ (mg.)	H ₂ O(mg.)	C%	H%
4.200	9.220	2.270	59.87	6.05
2.945	6.440	1.510	59.64	5.74
Average			59.75	5.89

To effect the hydrolysis of the pigment with strong sulphuric acid 50% H₂SO₄ was added to a small quantity of the sample and the mixture was boiled directly on the frame for 30 minutes until the solution gave a deep bluish-green colour, whereupon the solution was diluted with water and filtered. When the filtrate was shaken with an addition of ether, the ethereal solution turned dark yellowish-green and the aqueous solution gave an azure colour emitting strong red phosphorescence.

The ethereal solution was separated and concentrated, and when the ether was removed from it there remained a small quantity of a bluish-green residue. The residue was insoluble in water but dissolved easily in alcohol. Its alcoholic solution was dark yellowish-green and gave off red phosphorescence. When the spectrum of the solution was examined there were a thick line in the center of the red region, a thin one near the yellow region, 2 thin ones in the green region, and a thick one near the blue region. When concentrated hydrochloric acid was added to the alcoholic solution, it turned bluish green, and when concentrated nitric acid was added to it, deep violet layer appeared instantly with a blue or bluish-green layer directly under it, and when the solution was shaken it turned green gradually, then fading turned yellow and in a while suddenly caused hot boiling, and the alcohol effervesced to overflow with gaseous nitrogen oxide from the test tube.

Purification of the pigment

The brown pigment dissolved easily in alcohol while it was fresh, but it had a propensity to produce insoluble matter gradually. When a small quantity of it was infused in ether for a test, it was found

to contain some soluble pigment and the necessity of purification. Accordingly 25 g. of the pigment was extracted with ether for a week until no more soluble pigment was left undissolved. Then the residue was dissolved in alcohol and filtered to separate the insoluble matter; it was concentrated at a low temperature and under a low pressure meanwhile gaseous nitrogen being passed through the solution and a large quantity of ether was added to precipitate a pigment. The precipitate was subjected to the operations of solution in alcohol and precipitation with ether again and again and then it was dried in a desiccator *in vacuo* over sulphuric acid. 15 g. of a dark green powder (a) were obtained. The insoluble matter in alcohol was washed well with alcohol repeatedly and dried in the same manner as described above to the yield 4 g. of a brown powder (b). The ethereal solution was concentrated and when the ether was removed from it there remained a dark bluish syrup of which the yield was 0.5 g. (C).

(Properties of C). C was insoluble in water, easily soluble in alcohol; the solution gave a dark brown colour and emitted strong red phosphorescence. When its spectrum was examined 4 distinct absorptions in all were recognized: a thick absorption line in the center of the red region, a thin one near the yellow region, a thick one in the green region, and the broadest one between the bluish-green part and the blue region. This absorption spectrum was similar to that of phytochlorine produced by the hydrolysis of chlorophyll. C had no reducing property at all. Its alcoholic solution turned bluish-green when concentrated sulphuric acid was added and turned red or yellowish red when concentrated nitric acid was added. It must be noticed that the brown pigment before purification had the above-described properties in some measure and that this spectrum resembled somewhat that of the pigment produced by the hydrolysis with concentrated sulphuric acid.

(Properties of "a" and "b"). "a" and "b" had a little astringency. The alcoholic solution of "a" gave a dark yellowish-brown colour and emitted slightly red phosphorescence. When their spectra were examined, a wide strong absorption in the center of the red region and a weak but thick absorption between a bluish-green part and the blue region, two in all, are recognized. When the reaction of the alcoholic solution to concentrated acid was tested, the solution

turned bluish-green to conc. sulphuric acid, green to conc. hydrochloric acid and green-red to conc. nitric acid.

"a" and "b" dissolved in dilute alkaline solutions and the solution turned deep brown (at first brown). When they were dissolved in ammonium silver solution separately, "a" turned dark brown and was precipitated sooner than "b". Their other properties were compared as follows:

(Ash). "a" contained 1.32% of ash and "b" 0.74%. When the inorganic constituents of "a" and "b" were tested for by burning 1 g. of each sample, Na was present according to the flame test and when they were tested by the hydrochloric acid reaction, the Mg reaction was seen, but the presence of Ca, Fe, Al and Mn was not recognized at all.

(Phosphorus). The phosphate reaction was hardly recognized in both, but as it seemed "a" contained a little phosphate 2 g. of the sample were burned and 4 mg. of $Mg_2P_2O_7$ were obtained in the estimation.

(Nitrogen). When "a" and "b" were dissolved in alkaline solution and heated, no odour of amine was evolved. When they were distilled with zinc dust, the pyrrole reaction was evident. When nitrogen was estimated by KJELDAHL's method, the amount contained in anhydrous "a" was 1.36% and that contained in anhydrous "b" was 1.07%.

(Reducing property). When the reduction values of "a" and "b" were determined by the method described in the preceding section, that of "a" was 0.84 and "b" was 0.73.

(Fusion with potassium). When "a" and "b" were fused with potassium, they both showed the phloroglucine reaction.

(Hydrogenation). When a small quantity of the pigment "a" was dissolved in alcohol and a hydrogenation was carried out, a considerable absorption was evident for a long time. At first the alcoholic solution was viscous, but the viscosity was much decreased after the hydrogenation, and a close relation between unsaturation and viscosity was proven.

(Elementary constitution). The amounts of C and H contained in pigments "a" and "b" were as follows:

" a "

Quantities of material (mg.)	CO ₂ (mg.)	H ₂ O(mg.)	C(%)	H(%)
3.430	7.305	1.890	58.08	6.17
3.735	7.995	2.045	58.38	6.13
Average			58.23	6.15

" b "

Quantities of material (mg.)	CO ₂ (mg.)	H ₂ O(mg.)	C(%)	H(%)
3.190	6.760	1.680	58.80	5.89
3.540	7.535	1.830	58.05	5.78
Average			57.93	5.84

The elementary constitution of "a" and "b" were compared as follows:

C(%)	H(%)	O(%)	N(%)	Ash(%)	Phosphorus (%)
"a" 58.23	6.15	32.94*	1.36	1.32	0.05
"b" 57.93	5.84	34.42*	1.07	0.72	trace

* These were the numerical values obtained by subtracting the respective sums of the amounts of C, H, N and ash contained in "a" and "b" from 100.

(Hydrolysis with concentrated sulphuric acid). To 2 g., each of "a" and "b" 40 c.c. of 25% H₂SO₄ were added and the mixtures were separately heated directly on the fire and hydrolysed for 3 hours. Both the solutions turned at first a beautiful bluish-green colour which became gradually darker. As the hydrolysis was effected with difficulty and large quantities of black residue resembling charcoal were produced, the solutions were filtered.

Residues. On washing with water and drying of the residues the amounts of N contained in them were estimated and their reduction values were determined.

	N (%)	Reduction values
"a"	2.58	0.40
"b"	1.58	0.80

Filtrate. The colour of the filtrate of "a" was deep and that of "b" was light. When ether was added to both the filtrates, the ethereal layers turned dark yellowish-brown and emitted red phosphorescence. The aqueous solutions remained bluish-green, its red phosphorescence became stronger.

(Part dissolved in ether). When the above-mentioned ethereal solutions of "a" and "b" were washed with water and concentrated to remove ether, the solution of "a" produced a small quantity of a bluish-green residue and that of "b" produced a slight quantity of a residue. The residues were insoluble in water, but easily soluble in alcohol. When their spectra were examined, a wide absorption line in the center of the red region, a slender one near the yellow region and a broad absorption band occupying the space between the green and blue regions were evidently recognized. When concentrated nitric acid was added to the alcoholic solution various changes of coloration were brought about and at last the alcoholic solution effervesced and overflowed as described above. When the transitions of the absorption bands of the spectra meanwhile were examined, they were as follows:

Blue → violescent-blue → violet → reddish-violet → reddish-brown →
A B C
 yellowish-brown → yellow → effervescence.
D E

- A The thick absorption band in the center of the region moved somewhat toward the yellow region and a slender absorption line near the yellow region disappeared; the whole yellow region became slightly dark and the broad absorption band between the green and blue regions was transferred to the central part of the green region.
- B The absorption bands in the red region were unchanged; the absorption in the central part of the green region became lighter and turned as dark as the yellow region.
- C The thick absorption band in the red region weakened; the yellow region was dark as ever; the absorption band in the green region extended and a weak absorption band appeared between the blue and violet regions.

- D The absorption band in the red region moved toward the infra-red region; the yellow region was dark as ever and the broad absorption band between the blue and violet regions strengthened.
- E As the yellow colour increased its strength the absorption bands in the red region moved toward the infra-red region, the absorption band in the green region moved toward the ultra-violet region and the absorption band in the yellow region was unchanged. When the coloration turned yellow almost all the absorption lines disappeared.

When the transitions of the absorption bands were examined, they moved from the region of a long wave-length to that of a short wave-length and their colour turned gradually lighter as the reaction proceeded. It seemed that this showed a decrease in the molecular weight accompanying the hydrolysis.

To interpret the above-described results; it is known that the brown pigment obtained by the saponification of δ was mixed with the hydrolysed products of chlorophyll (mainly chlorophyll a) as δ was hydrolysed with a large quantity of chlorophyll unlike the other cases (those of α , β and γ), and the separation of these hydrolysed products seemed to be pretty difficult, for some quantity of a pigment soluble in ether was contained in the pigment which should have been considerably purified even after it was separated and dried. This pigment (C) was similar to phytochlorine "e" derived from chlorophyll "a", as its spectrum showed. What is doubtful is that the spectrum of the pigment (C) was similar in some points to that of a small quantity of a pigment which was produced in the course of the hydrolysis of the brown pigment with concentrated sulphuric acid. It is not clear whether this pigment arose from the decomposition of the brown pigment or it was an impurity mixed in the brown pigment. When the results of the elementary analysis were examined the percentage of carbon of the pigment before purification was higher than after purification, and this perhaps was because the amount of the substance having higher percentage of carbon which was mixed in the substance dissolved in ether was large.

When the pigments "a" and "b" separated as two sorts in the purification were examined, "a" contained more ash and somewhat less oxygen than "b", and a slight quantity of phosphorus. Both

"a" and "b" gave the pyrrole and phloroglucine reactions and had a reducing property. Generally speaking, it seems to have the same properties as the pigments obtained from the other brown substrata. When hydrogenation was carried out for the test of the manifestly a property of absorbing hydrogen. It is to be noticed that the alcoholic solution of the pigment had considerable viscosity like alkaline solutions, and the viscosity was much decreased by hydrogenation. This fact suggests that there is a close relation between unsaturation and viscosity, and this property is just the same as in other pigments.

2) Biological properties of the brown pigment

Formerly the author isolated a dark green pigment from the fat of *Hizikia fusiforme* (HARV.) OKAM. As it was known that a part of it dissolved in water and the solution produced crystals after long standing, a large quantity of the pigment dissolved in water was allowed to stand in a flask covered with paper for 3 years, and wonderful to say the surface of the solution was not with any mould at all. On the suggestion of that fact the author thought something of the same sort might occur with the pigment newly isolated by him. However, it was learned by tests to the author's surprise, that the growth of aerophile mould was promoted by the brown pigment and that of aerophobe lactic acid bacteria was restrained by the same. These tests will be described in the following paragraphs.

The moulds used in the tests were *Penicillium chrysogenum* THOM and *Aspergillus ochraceus* WILHELM and the lactic acid bacteria used were *Lactobacillus bulgaricus* (LUERSSEN and KÜHN) HOLLAND and *Lactobacillus helveticus* (ORLA-JENSEN) BERGEY et al. (All microbes were obtained from American type culture collection).

A definite quantity of each of the dilute alkaline solutions of various concentrations of the brown pigment was added to the culture media and the state of growth of the microbes was observed comparatively.

Test of moulds. 0.01 g., 0.03 g., 0.05 g. and 0.10 g. of each of the brown pigments "a" and "b" were dissolved in 5 c.c. of N/5 NaOH solution respectively. One c.c. of each of these solution was added to 9 c.c. of CZAPEK's solution agar and the mixture was poured into a Petri dish and inoculated with mould. Besides for control 1 c.c. of N/5 NaOH solution not containing the pigment or 1 c.c. of

water was added to 9 c.c. of the culture medium and was inoculated. So the degree of alkalinity of the culture medium was N/50 and the concentration of the pigment contained was 0.02%, 0.10% and 0.20% respectively. The diameter of the colony was measured and the degree of growth was examined at room temperature every fourth day and the results were as described next.

Taking these results under consideration, it was evident that the growth of each mould did not differ so much for the first week or thereabouts whether it was cultured on the culture medium containing the pigment or on that without the pigment (control), but afterwards the growth of the mould cultured on the former medium was considerably promoted. The differences induced by the pigments "a" and "b" and their various concentrations were not very distinct, but generally speaking, to a certain extent, it seemed the higher the concentration of the pigment the more developed the growth of the mould. The positions of the figures in the diagrams show roughly the degrees of the promotion of the growth.

TABLE VII. The effect of the brown pigments of various concentrations on the growth of the moulds.

Kind of Day Culture medium	P. chrysogenum							Asp. ochraceus							
	4	7	10	13	17	19	21	4	7	10	13	17	19	21	
H ₂ O	47	80	98	98	98	113	150	52	61	70	105	115	130	140	
Control	47× 55	70× 90	85× 95	88× 100	100× 110	100× 125	155	30	33	37	58× 65	70	76	90	
a	1 0.02%	47	75	104	140	182	190	180× 200	30	38	40	75	85	97× 105	105
	2 0.06%	47	77	104	140	190	206	208	35	35× 45	55× 48	80× 90	104× 95	115	115× 130
	3 0.10%	47	80	113	123	125	145	185	35	40	54	80	110	112	130
	4 0.20%	45	73	105	145	195	205	206	32	35	48	80	110	120	145
b	1 0.02%	47	75	104	140	187	200	215	30	33	40	67	90	95	100
	2 0.06%	47	78	107	145	198	216	220	32	36	55	75	100	105	115
	3 0.10%	43	78	107	150	190	216	230	35	40	55	85	110	112	130
	4 0.20%	48	83	107	135	154	180	200	35	35× 40	55	80× 90	110× 102	110× 117	130× 140

N.B. The degrees of the growth are represented by figures, 3.03 cm. corresponding to 100. 34×40 represents the length and breadth of a colony. H₂O represents the culture medium added water instead of the alkaline solutions of the pigments. Control means a culture medium not containing the pigment.

Fig. 10

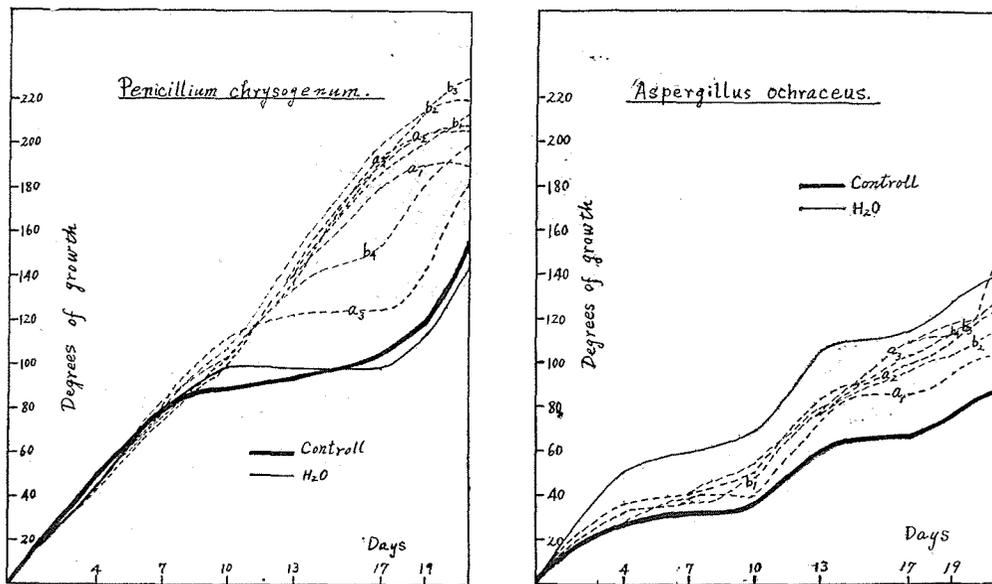
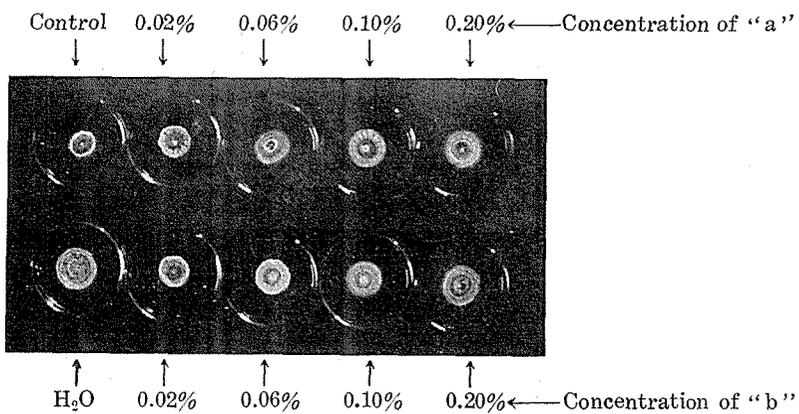
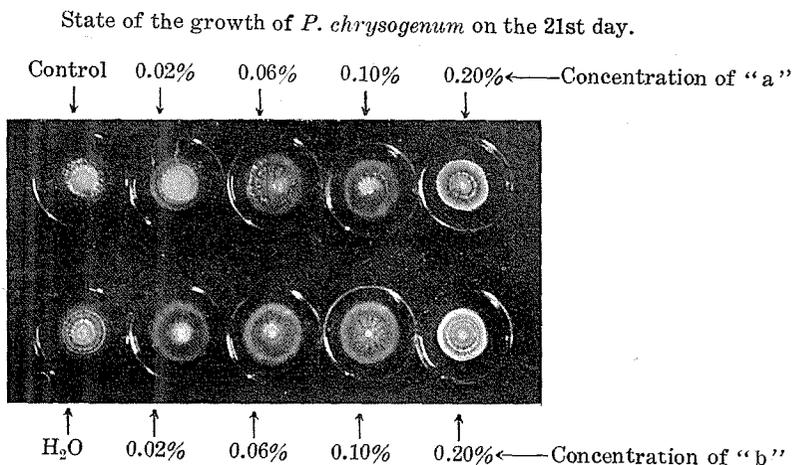


Fig. 11

State of the growth of *Asp. ochraceus* on the 21st day.





Test of lactic acid bacteria. The culture medium was made by the following procedure.

Skimmilk was heated to 60°C. and on precipitation of casein with an addition of lactic acid it was filtered. The filtrate was neutralised with sodium carbonate, heated and filtered again to be made translucent. To 2 l. of the whey thus obtained, 200 c.c. of the trypsinized casein solution were added and the mixture was used as the fundamental culture medium.

Trypsinized casein solution: 10 g. of casein were dissolved by warming in 100 c.c. of 0.1% sodium carbonate solution, and after cooling to 40°C., 0.5 g. of 100,000 unit trypsin manufactured by Kahlbaum and Co. was added to the solution and moreover 10 c.c. of chloroform; digestion was allowed to proceed at 37°C. for 48 hours under air-tight plugging. Then the liquid was neutralised with hydrochloric acid and heated to expel the chloroform and to reduce the liquid to 100 c.c.

To every 99 c.c. of the medium was added 1 c.c. of each pigment solution described above. Therefore the concentration of the pigment in this culture liquid corresponded to one tenth of the amount of the pigment contained in that of the moulds. Ten c.c. of each culture liquid thus prepared were taken in a test tube separately and after inoculation were incubated at 37°C., and then in every test 5 c.c. were taken and titrated with N/20 NaOH solution.

The results of these operations were as follows:

TABLE VIII. Effects of the brown pigments of various concentrations on the growth of lactic acid bacteria. (The titration numbers of N/20 NaOH solution required for 5 c.c. apiece of various culture media)

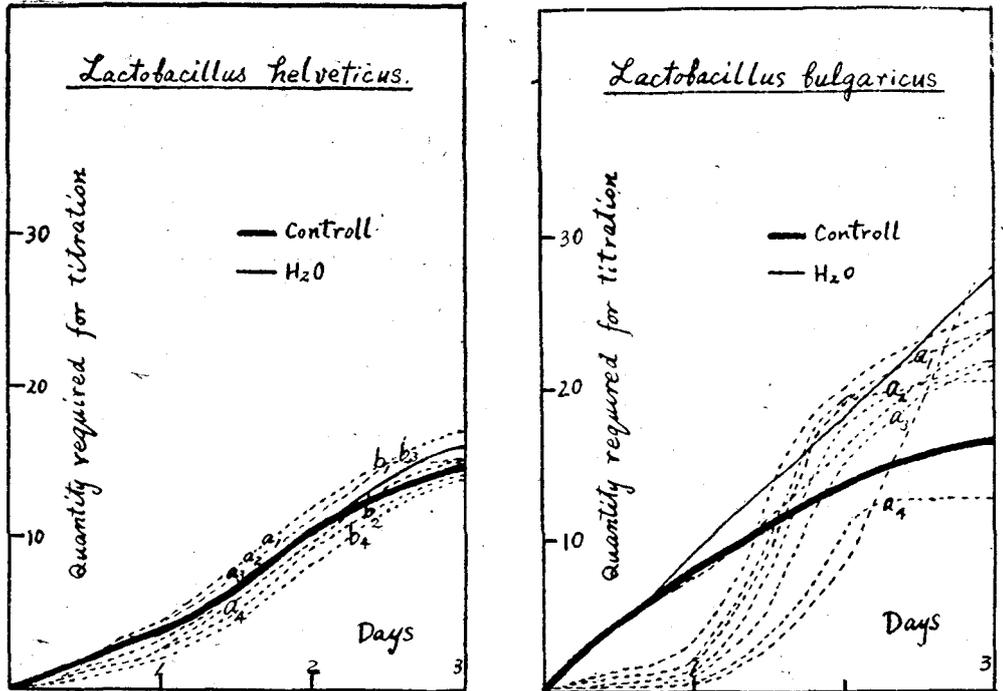
Lactobacillus helveticus

Culture media	Days	0		1		2		3		6	
		Titration number	Tit. no.	In-creases	Tit. no.	Inc.	Tit. no.	Inc.	Tit. no.	Inc.	
H ₂ O controls		2.31	0.71	4.40	15.58	11.27	18.28	15.97	20.74	18.43	
		1.98	5.69	3.71	12.94	10.96	17.12	15.14	20.62	18.64	
a	0.002%	1.68	4.32	4.64	13.55	11.87	18.56	16.88	19.70	18.02	
	0.006%	1.85	5.85	4.00	12.90	11.05	17.96	16.11	20.74	18.36	
	0.010%	2.39	4.45	2.06	12.61	9.22	17.78	15.39	18.92	16.53	
	0.020%	2.49	4.89	2.40	12.26	9.77	16.92	14.43	21.42	18.93	
b	0.002%	2.11	4.76	2.65	12.91	10.80	17.88	15.97	20.70	17.59	
	0.006%	2.16	5.10	2.94	12.56	10.40	17.72	15.56	20.50	18.34	
	0.010%	2.19	4.81	2.62	12.31	10.12	16.64	14.45	19.72	17.53	
	0.020%	2.34	5.71	3.57	11.41	9.07	16.60	14.26	18.30	15.96	

Lactobacillus bulgaricus

Culture media	Days	0		1		2		3		6	
		Tit. no.	Tit. no.	Inc.	Tit. no.	Inc.	Tit. no.	Inc.	Tit. no.	Inc.	
H ₂ O controls		2.31	12.09	9.76	20.46	18.11	29.98	27.67	35.52	33.21	
		1.98	10.43	8.45	16.13	14.15	28.60	16.62	34.50	32.52	
a	0.002%	1.68	3.05	1.37	19.86	18.18	23.70	22.02	32.96	31.28	
	0.006%	1.85	3.26	1.41	20.48	18.63	25.70	23.85	31.60	29.77	
	0.010%	2.39	3.38	1.01	17.43	15.04	19.50	17.11	20.08	17.69	
	0.020%	2.49	3.41	1.92	13.74	11.25	15.52	13.08	12.76	10.27	
b	0.002%	2.11	9.73	7.62	18.56	16.45	27.04	24.93	35.22	33.11	
	0.006%	2.16	4.48	7.32	20.76	18.60	23.26	21.10	31.84	29.68	
	0.010%	2.19	3.38	1.19	21.35	9.16	30.40	28.21	33.22	31.03	
	0.020%	2.34	5.21	2.87	21.85	19.51	27.50	25.16	34.42	32.08	

Fig. 12



When these results were examined, it was seen that all lactic acid bacteria seemed to tend, generally speaking, to be restrained their growth in contrast to moulds. The growth of *Lactobacillus bulgaricus* was promoted after 2 days. Perhaps this was because the pigments were precipitated by the acid in the culture liquids of which the alkali solution required for titrations were increased over 15 c.c and consequently the action of the pigments in the culture liquids was weakened, suddenly promoted the growth of the bacteria. The pigments in the culture liquids of *Lactobacillus helveticus* were always dissolved and consequently their effects seemed to work even after 2 days.

The differences induced by the pigments "a" and "b" were indistinct as in the case of moulds, and as to the differences caused by the various concentrations of the pigments, generally speaking, the thicker the culture liquids, the stronger was the restraint of the growth of the bacteria, as may be seen by comparing the positions of the figures in the diagrams.

3) Summary

When the properties of the brown pigment obtained by the saponification of the brown substratum δ described in the preceding section were examined, they were, generally speaking, the same as those of the brown pigments obtained by the saponification of α , β and γ , and as a rather large yield was obtained in this case some experiments such as had not been done before were carried out.

In this case a substance soluble in ether like phytochlorine "e" derived from chlorophyll "a" was contained in the brown pigment and was not easily separable and as a part of the pigment turned insoluble in alcohol the pigment was divided into the pigments "a" and "b" and the properties of both were examined. As to the elementary compositions pigment "b" contained a little more oxygen than "a", and the former was more sensitive to oxidation than the latter. Further, pigment "a" contained a little more ash than pigment "b" and when the constituents of the ash were investigated, Mg occupied a large part and Na was small. When pigment "b" was dissolved in alcohol and examined to see whether the pigment was hydrogenated or not, it was evident that the pigment absorbed a small quantity of hydrogen after a long time. At the same time it was recognized that the solution which was considerably viscous decreased its viscosity after hydrogenation. Therefore it was known that a close relation existed between unsaturation and viscosity. When each pigment was hydrolysed with concentrated sulphuric acid, a small quantity of a substance soluble in alcohol was produced in each case, pigment "a" producing more than pigment "b". This substance gave the various colour reactions by concentrated nitric acid and after a while the reaction becoming vigorously showed the phenomenon of effervescence. The spectrum of this substance was somewhat similar to that of phytochlorine "e". When the estimation of nitrogen and the reduction value of the residues from which this substance was removed, were carried out, their reduction values were decreased strikingly and the amounts of nitrogen were increased. This was perhaps because the percentage of nitrogen of the nitrogenous part increased, as the part possessing reducing property was removed. The pyrrole and phloroglucine reactions of the pigments were like the case described in the preceding section. Further, the special influences of pigments "a" and "b" on the

growth of microbes were recognized. They promoted the growth of moulds and restrained that of lactic acid bacteria.

C. Consideration

When the properties of the phosphorous compounds separated as the brown substrata of the *Phaeophyceae* were examined, these substances behaved toward the various solvents as if they were fats (γ) and (δ), of cerebroside (α), or of lecithin (β). At first they were very hydrophile and had a strong tendency to form hydrosol, but they gradually lost these properties and lowered their solubility in the solvents as fats or lipoids do. It is not without reason that they were once looked upon as fatty substance. It is not clear that these substances were substantially quite different or that they behaved differently toward solvents by physical and chemicas changes although they were substantially the same, but γ and δ seemed to be in a close relation with one another. According to KYLIN's investigation, the distribution of the physoden coincided with that of lipoids and on this point the important significance of these substances must be recognized. When these substances were saponified, there were produced glycerine, phosphorous compounds, fatty acids (?) and trimethylamine besides a large quantity of the brown pigments. The brown pigments will be spared description, but the easy emission of the odour of amine immediately after the saponification of the brown substrata demands a little attention. It is not yet quite clear the decomposition of what base is the cause of the phenomenon, but it is thought that the combination of amine itself might be the cause. The nonvolatile base feaction was faint in the solution in which the saponification had been carried out and the presence of the nonvolatile base is very dubious. The presence of trimethylamine as volatile base and stachydrine⁽⁹⁾⁽³¹⁾ and choline⁽³⁶⁾ as nonvolatile bases in the seaweeds has hithertofore been known. But their sources were not yet elucidated. As to the bases⁽²⁶⁾ of *Cystophyllum hakodatense* YENDO, although the presence of trimethylamine as volatile base was recognized, the presence of choline, betaines and stachydrine as nonvolatile bases was not recognized as formerly mentioned by the present author. When these facts are taken into consideration, it is thought that the source of amine may be the brown phosphorous compounds separated by author.

With respect to the presence of fatty acids, notwithstanding the fact that only impure compounds were obtained, it is not reasonable absolutely to deny its presence. This point has to be resolved by future researches.

Then, when the properties of the brown pigments are considered, although these pigments are not necessarily alike according to the conditions of the hydrolysis as described above (on this point they resemble the hydrolysed products of chlorophyll), they all seem to stand qualitatively in close similarity no matter in what case they may have been produced.

The chemical properties of the various products are compared as follows:

The reduction values determined of β and γ were not exact as the bubbling up of the soap at the determination was vehement owing

Amounts of phosphorus and nitrogen contained in the various brown substrata and brown pigments and the reduction values of them.

	P	N	Reduction values
α	0.25%	1.39%	0.95
β	0.25	1.50	0.20 (?)
γ	0.27	1.08	0.30 (?)
δ	+	+	Not clear
α	5 hours hydrolysis with 3% baryta water	trace	—
	5 hours hydrolysis with 5% sulphuric acid and 8 hours hydrolysis with 10% hydrochloric acid	„	1.40
	5 hours hydrolysis with 5% sulphuric acid at 150°C	„	1.48
β	3 hours hydrolysis with 5% baryta water	„	—
γ	3 hours hydrolysis with 5% baryta water	„	0.91
	4 hours hydrolysis with 5% sulphuric acid	slight quantity	1.40
δ	1 hour hydrolysis with 10% NaOH (a)	0.05	1.36
	1 hour hydrolysis with 10% NaOH (b)	trace	1.07
	3 hours hydrolysis of (a) with 25% H ₂ SO ₄	„	2.58
	3 hours hydrolysis of (b) with 25% H ₂ SO ₄	„	1.58

to the fats contained in them. It seemed that those brown pigments which were advanced in decomposition had generally low reduction values, and contained, on the contrary, large amounts of N.

This tendency was manifest when the brown pigments "a" and "b" were vigorously hydrolysed with strong acids. Perhaps as the parts having the carbonyl radicals dissolved away; the amounts of N contained in the remaining pigments became larger. However, the amounts of N contained in the brown pigments being adequately decomposed and liberated amounted generally to about 1.4%. This was perhaps because of the pyrrole nuclei. In this respect the brown pigments are similar to chlorophyll and haemoglobin. This fact compared with the fact that of late years the presence of the pyrrole in seaweed pigments as phycoerythrin and phycocyan⁽¹⁴⁾ has been established, is very interesting. It was not necessarily absurd that COHN, the first researcher on the brown pigments thought that they were perhaps connected with chlorophyll. However, as the brown pigments give the phloroglucine reaction by infusion with potassium they seem to be of tannin quality when considered from this point. Further, they have reducing properties and unsaturated nature not arising from saccharides, and are very similar to the tannin pigments of the barks and others as TAKEO TAKAHASI⁽²⁸⁾ pointed out. His elementary analysis of his dark brown pigment compared with the results of the elementary analysis of the pigments "a" and "b" produced by the author are as follows:

TAKAHASI's dark	C%	H%	O%	N%	Ash %
brown pigment ...	61.04	5.00	33.95	0	0
"a"	58.23	6.15	32.94	1.36	1.32
"b"	57.93	5.84	34.42	1.07	0.74
Pigment before separation into "a" and					
"b"	59.75	5.89	31.81	1.28	1.27

TAKEO TAKAHASI stated that, as a small amount of an insoluble substance was produced when he estimated the molecular weight of the pigment mentioned above it was removed and the filtrate was purified by repeating evaporation to dryness. The substance obtained by the purification seems to correspond to the pigment "a" obtained by the author.

As to the properties of the brown pigment "a", it demands a little attention that the viscosity of its alcoholic solution decreases remarkably on hydrogenation. The facts suggest that a close relation exists between the unsaturation and the viscosity of the pigment. This is an interesting property of the pigment from the view-point of biochemistry.

These pigments exert special influences upon the growth of moulds and lactic acid bacteria, promoting the growth of the former and restraining the growth of the latter. Although it is not clear these influences seem to have some relation with the oxidation of the pigments.

As to the phosphatide of *Alaria crassifolia* KJELLM. a description was given in the preceding chapter. No report has previously been published on the phosphorous compounds of this sort. They seem to be substances peculiar to seaweeds. The author proposes to call these substances phycophosphatides and desires to keep for them the term "phycophaine" used hithertofore for the brown pigments contained in the phosphatides.

VI. Résumé

In this paper the author has treated the properties of several substances newly separated as constituent elements, unsaponifiable matter and phosphatide of marine algae fat, mainly from the view-point of the physiological functions.

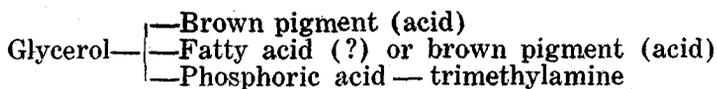
A new sterol was separated by the present author which was styled pelvesterol. The chemical properties of pelvesterol agreed well with fucosterol which has been studied on its chemical constitution by HEILBRON and his collaborators. Accordingly, in the present paper attention is given to the observation of the nature of the physiological functions of pelvesterol and the elucidation of the relations between its chemical constitution and its physiological functions. It was noted that pelvesterol has a special activating function for the action of lipase, and this activating property has a close relation with unsaturation and with the phenolic nature of sterol. Consideration was given to the fact that the lipase-activating function of sterol differs according to the number and position of the double bonds contained in the phenanthrene nucleus and the kinds of acids closed OH radical. It was noted that the curve of the lipase-activat-

ing function of pure pelvesterol represents a definite figure and therefore it will be of use to employ the method in the study of sterols of the kind.

It was ascertained that there are some substances that activate the action of lipase other than pelvesterol in the liquid constituents of unsaponifiable matter of fats. Some of them have cramp and narcotic toxicity similar to that of the toxic element of liver oil, and this character varies according to the part of plants from which they come. The existence has been noted of substances which have the reactions of unsaturated hydrocarbons such as $C_{18}H_{36}$, $C_{21}H_{38}$ and $C_{20}H_{34}$ and hydrocarbons of the terpene group. These compounds have been rarely known in fish oils and are found for the first time in seaweeds.

A crystal having the empirical formula $C_7H_{10}O_2$ has been isolated in which no activating property for the function of lipase and no toxicity can be recognized. This is thought to be a special compound, but its properties have not yet been ascertained.

Special phosphatides have been isolated. One of them is a cephaline-like substance having the characteristic of containing a higher unsaturated fatty acid. Another is a substance which is the brown substratum of *Phaeophyceae* and on hydrolysis easily gives amine and liberates a large quantity of brown pigment. The existence of glycerine, phosphoric acid, fatty acid (?) etc. in it, was recognized but not that of a nonvolatile base. Its saccharide reaction is negative. Perhaps its original may be a mixture of substances taking a form similar to a phosphatide such as this:



As to the trimethylamine, the author presumes that it may be supplied from this substance. With regard to brown pigment TAKEO TAKAHASI has already made a valuable study, but he did not touch upon the combination form. The present author has found that the brown pigment has the functions to promote the growth of mould and restrain the growth of lactic acid bacteria.

As no one has ever isolated from seaweeds such a phosphatide, the author proposes here to give it the name "phycophosphatide" and to apply to the brown pigment the name "phycophaine" which has been used heretofore.

Literature

- (1) BENGTSOON, B. E.: Ein Vergleich zwischen Sitostanol und Stigmastanol. *Z. physiol. Chem.*, *237* 46-51 (1935).
- (2) BERGMANN, W.: Contributions to the study of marine products. II. The sterols of mollusks. *Biol. Chem.*, *104* 317-328 (1934).
- (3) COFFEY, D. H., HEILBRON, I. M. and SPRING, F. S.: Studies in the sterol group. Part XXIII. The location of the ethenoid linkage of α -dehydrofucosterol and the identity of sitostanol, fucostanol, stigmastanol and ostreastanol. *J. Chem. Soc.*, 738-741 (1936).
- (4) HAAS, P. and RUSSELL-WELLS, B.: : On the significance of the ash content of certain marine algae. *Biochem. J.* *17* 696-706 (1923).
- (5) HAAS, P. and RUSSELL-WELLS, B.: A note on the phosphorus content of marine algae. *Biochem. J.* *29* 1915-1917 (1935).
- (6) HEILBRON, I. H., PHIPPERS, R. F. and WRIGHT, H. R.: Chemistry of the brown algae. *Nature*, *133* 419 (1934).
- (7) HEILBRON, I. H., PHIPPERS, R. F. and WRIGHT, H. R.: The chemistry of the algae. Part. I. The algal sterol fucosterol. *J. Chem. Soc.*, 1572-6 (1934).
- (8) KAWAKAMI, K. and YAMAMOTO, I.: On the toxic components of fish liver oils. *J. Agr. Chem. Soc. Japan*, *11* 400-421 (1935).
- (9) KLEIN, G. u. LINSER, H.: Zur Bildung der Betaine und der Alkaloide in der Pflanze. I. Die Bildung von Stachydrin u. Trigonellin. *Z. physiol. Chem.*, *209* 75-102 (1932).
- (10) KOGANEI, R.: On fatty acids obtained from cephalin. compounds of β -aminoethyl alcohol with saturated and unsaturated fatty acids. *J. Biochem. (Japan)*, *3* 15-26 (1924).
- (11) KYLIN, H.: Ueber die-Farbstoffe der Fucoideen. *Z. physiol. Chem.*, *82* 221-230 (1912).
- (12) KYLIN, H.: Zur Biochemie der Meeresalgen. *Z. physiol. Chem.*, *83* 171-197 (1913).
- (13) KYLIN, H.: Untersuchungen über die Biochemie der Meeresalgen. *Z. physiol. Chem.*, *94* 337-425 (1915).
- (14) KYLIN, H.: Einige Bemerkungen über Phykoerythrin und Phykocyan. *Z. physiol. Chem.*, *197* 1-6 (1931).
- (15) LEVENE, P. A. and ROLF, I. P.: The glyserphosphoric acid of cephalin. *J. Biol. Chem.*, *40* 1-16 (1919).
- (16) LEVIN, V. E. and RICHMANN, E.: Reactions of terpenes with antimony trichloride. *Bioche. J.* *27* 2051-4 (1913).
- (17) MATSUOKA, M.: On cramp caused by an over-dose injection of biosterol. *Bull. Inst. Physic. Chem. Res.* *13* 1267-1283 (1934).
- (18) Cited from OLTMANN'S "Morphologie u. Biologie der Algen." Band III., S. 46 (1923).
- (19) PARNAS, J.: Ueber die gesättigte Fettsäure des Kephals. *Biochem. Z.* *56* 17-20 (1913).

- (20) ROSENTHALER, L.: "Der Nachweis organischer Verbindungen Ausgewählte Reaktionen u. Verfahren." s. 14-15 Stuttgart (1923).
- (21) RUSSELL-WELLS, B.: Fats of brown sea-weeds. *Nature*, 129 654-655 (1932).
- (22) SHIRAHAMA, K.: "On the unsaponifiable matter of algae fats." Announced in a lecture-meeting of Sapporo Society of Agriculture and Forestry at 7th Oct. (1933).
- (23) SHIRAHAMA, K.: "On the unsaponifiable matter of the algae fats." I. *Sterol. J. Agr. Chem. Soc. Japan*, 11 980-984 (1935).
 ———: II. Pelvesterol from *Hijikia fusiforme* (HARV.) OKAM. *J. Agr. Chem. Soc. Japan*, 12 521-522 (1936).
 ———: III. On the toxic components. *J. Agr. Chem. Soc. Japan*, 13 13 705-9 (1937).
 ———: IV. On the lipase activation as influenced by pelvesterol. *J. Agr. Chem. Soc. Japan*, 14 349-351 (1938).
 ———: V. On the "activity curves" for lipolytic action and the spectrum of pelvesterol. *J. Agr. Soc. Japan*, 14 415-420 (1938).
 ———: VI. On some activators for lipolytic action. *J. Agr. Chem. Soc. Japan*, 14 421-422 (1938).
 ———: VII. Some new ingredients of brown algae (This subject is continued from preceding reports entitled "On the unsaponifiable matter of algae fats") VII. On the unsaponifiable liquid part of the algae fats. *J. Agr. Chem. Soc. Japan*, 14 743-748 (1938).
 ———: VIII. On the brown compounds of phosphorus. *J. Agr. Chem. Soc. Japan*, 14 1325-1331 (1938).
 ———: IX. A brown pigment. *J. Agr. Chem. Soc. Japan*, 14 1397-1403 (1938).
 ———: X. Brown phosphorus-containing compounds and brown pigments. *J. Agr. Chem. Soc. Japan*, 14 1433-1436 (1938).
- (24) ———: On the phosphatide of sea-weeds. *Sapporo Soc. Agr. and Forest. (Japan)*, 29 40-50 (1937).
- (25) ———: On the bases of sea-weeds. *Sapporo Soc. Agr. and Forest. (Japan)*, 29 234-7 (1937).
- (26) SUMI, M.: On the sterin isolated from several vegetables. *Bull. Inst. Physic. Chem. Res.*, 8 228-232 (1929).
- (27) TAKAHASHI, E., SHIRAHAMA, K. and TASE, S.: Chemical studies on fats of algae. I. On the contents of fats and their properties, especially on the fatty acid of *pelvetia wrightii* YENDO. *J. Chem. Soc. Japan*, 54 619-623 (1933).
 TAKAHASHI, E., SHIRAHAMA, K. and TASE, S.: On the fats of sea algae. II. *J. Chem. Soc. Japan*, 56 1250-1257 (1935).
 TAKAHASHI, E. and SHIRAHAMA, K.: On the fats of sea algae. III. On the fats of *Hijikia fusiforme* (HARV.) OKAM. *J. Chem. Soc. Japan*, 57 411-414 (1936).

- TAKAHASHI, E., SHIRAHAMA, K. and ITO, N.: On the fats of sea algae. IV. On the fatty acids of *cystophyllum hakodatense* YENDO. J. Chem. Soc. Japan, 59 662-666 (1938).
- TAKAHASHI, E., SHIRAHAMA, K. and TOGASAWA, Y.: On the fats of sea algae. V. On the fatty acids of *Alaria crassifolia* KJELLM. J. Chem. Soc. Japan, 60 56-60 (1939).
- TAKAHASHI, E. and SHIRAHAMA, K.: On the fats of sea weeds. J. Japanese Assoc. for the advancement of Science, 14 267-271 (1939).
- (28) TAKAHASHI, T.: On the studies of Kajime (*Ecklonia cava* KJELLM.) III. Report of Tokyo Exper. Stat. Ind. pp. 1-10 (1931).
- (29) TADOKORO, T. and ABE, M.: On the chemical studies of sea weeds (Japanese). Report of Muroran Inst. Res. Sea-weeds, Hokkaido Imp. Univ., 2 80-81 (1933).
- (30) TAKASE, T.: "Physiological action and chemical structure" (Japanese text) p. 521 (1925).
- (31) TODA, S.: Beiträge zum biologischen Studium der organischen Basen. II. (Dritte Mitteilung). J. Biochem. (Japan) 433-6 (1923).
- (32) TSUJIMOTO, M.: New method for fish oil test. J. Soc. Chem. Ind. Japan, 29 561-4 (1926).
- (33) TSUJIMOTO, M.: J. Soc. Chem. Ind. Japan, 38 673 (1935).
- (34) TSUJIMOTO, M.: "On the studies of liver oil" (Japanese) pp. 94-102 (1936).
- (35) UENO, S. and IWAI, S.: J. Soc. Chem. Ind. Japan, 35 1461-5 (1932).
- (36) ZELLER, A.: Ueber das Vorkommen von Cholin in Meeresalgen. Biochem. Z., 268 187-8 (1934).
- (37) NORD, F. F. und WEIDENHAGEN, R.: "Ergebnisse der Enzymforschung." Band VI s. 184, Leipzig (1937).