

Phenolic Compounds in Brown Algae

I. The Presence of Reducing Compounds in *Ascophyllum nodosum*(L.)Le Jol.

ARNE HAUG and BJØRN LARSEN

Norwegian Institute of Seaweed Research, N.T.H., Trondheim, Norway

The brown alga *Ascophyllum nodosum* contains amounts of reducing compounds varying from 0.4 to 2.25 mequiv. per g dry matter depending on place of growth. Evidence is presented which indicates that the reducing compounds show reactions characteristic of phenols, and that they are identical with the compounds giving the color reaction of the physodes ("fucosan").

It has been known for more than sixty years that the cells of brown algae often contain a large number of highly refractive, colorless vesicles which are usually termed physodes. In 1893 Crato¹ showed that the physodes could reduce silver nitrate, and that they could be stained by vanillin hydrochloric acid. From this and other evidence he concluded that the physodes contained phenols, and that these phenols consisted partly, at least, of phloroglucinol. His results were later confirmed by several authors, among them Kylin^{2,3} who described the contents of the physodes as compounds related to tannins. He noticed that these tannin-like compounds were rapidly oxidised to brown colored substances under alkaline conditions. In a note from this laboratory⁴ it was shown that a diazo reagent could be used to distinguish between *Laminaria hyperborea* (Gunn.) Foslie and *Laminaria digitata* (Huds.) Lamour., and later, unpublished work showed that the diazo reagent colored specifically the physodes.

In 1943 Rønnerstrand⁵ reported that brown algae contained a compound that interfered in the titration of vitamin C with dichlorophenol-indophenol, and he assumed that this reducing component ("fucosan") was contained in the physodes. He also attempted a quantitative estimation of the "fucosan" content by titration with dichlorophenol-indophenol in dilute metaphosphoric acid after vitamin C was destroyed by means of ascorbinase. In *Ascophyllum nodosum* he found a "fucosan" content corresponding to 18—32 mg ascorbic acid per 100 g fresh algae.

In 1957, Esping⁶ reported that the enzyme inhibitory effect of extracts from *Fucus vesiculosus* L. probably was due to a polyphenol.

It was observed in this laboratory that the acid extracts of dried *Asco-phyllum nodosum* contained considerable amounts of reducing compounds, while the reducing power of similar extracts of dried *Laminaria* species was insignificant. It was shown that it was possible in this way to distinguish between meal produced from Fucaceae and Laminariaceae species. Extracts of fresh *Laminaria* species, on the other hand, showed a considerable reducing power. The difference is thus due to a loss of reducing power during drying of *Laminaria* species, and will be subject to further investigation.

The present investigation is confined to the reducing compounds of *Asco-phyllum nodosum*.

EXPERIMENTAL

Extraction. Extracts of fresh material were prepared in the following way: 40 g fresh algae was disintegrated with 200 ml 0.2 N sulfuric acid in a Waring blender. Solid particles were removed by centrifuging.

When dry material was used, 1 g ground algae was extracted with 50 ml 0.2 N sulfuric acid overnight, and the extract was filtered.

Potentiometric titration. 10 ml extract was neutralised with approximately 1 ml 2 N sodium hydroxide. After addition of 10 ml 0.5 N sodium hydroxide, the extract was titrated by batchwise addition of 0.01 N potassium ferricyanide solution in 0.25 N sodium hydroxide. The solution was stirred vigorously throughout the titration. Near the end-point of the titration, the reaction proceeds slowly, and the solution must be allowed to stand for about 5 min before the equilibrium potential is reached. The potential was measured against a saturated calomel electrode.

The relation between the potential and pH was determined in the following way: 10 ml extract was made alkaline with 2 ml 2 N sodium hydroxide. A 0.05 N solution of potassium ferricyanide in water was added in a quantity sufficient to oxidise half the amount of reducing components present. After 5 min 1 ml 2 N hydrochloric acid and 10 ml buffer solution of the desired pH were added and the potential was measured.

Color reactions. The vanillin reagent was prepared by dissolving 5 g vanillin in a mixture of 20 ml conc. sulfuric acid and 10 ml water. 1 ml extract and 5 ml reagent were thoroughly mixed and after about half an hour the red color was measured in a Beckman DU spectrophotometer at 515 $m\mu$. Extracts with a high content of phenolic compounds formed a colored precipitate with the reagent, and such extracts were therefore diluted before the reagent was added.

The diazo reagent was prepared by dissolving 100 mg diazosulfanilic acid in 20 ml 3 % sodium carbonate solution. 2 ml reagent was then mixed with 2 ml extract diluted with sufficient 3 % (w/v) sodium carbonate solution to give a color intensity which could be measured directly. The optical density at 470 $m\mu$ was then determined. As a blank was used 2 ml reagent mixed with 2 ml 3 % sodium carbonate solution. The reagent is unstable and the measurements must be carried out as fast as possible.

The brown color in alkaline solution was obtained by mixing 1 ml extract with 4 ml 3 % sodium carbonate solution and heating the mixture for 15 min at 120°C. The color was measured at 420 $m\mu$.

Determinations with hide powder. 20 g dried seaweed sample was extracted with 1 000 ml 40 % (v/v) aqueous acetone overnight. The acetone was removed *in vacuo*, and water was added to give 1 000 ml. 50 ml of this extract was used for determination of dry matter and ash. To 200 ml extract acidified with 5 drops conc. hydrochloric acid was added 100 g hide powder *. After 24 h 50 ml of the filtered solution was used for determination of ash and dry matter. The amount of "tannins" is taken as the organic dry matter removed by the hide powder treatment.

* Hide Powder p.a. Merck 4332.

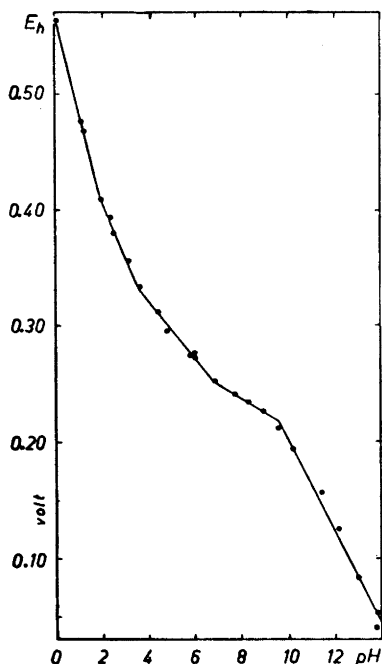


Fig. 1. pH dependence of the potential of extracts from *Ascophyllum nodosum*.

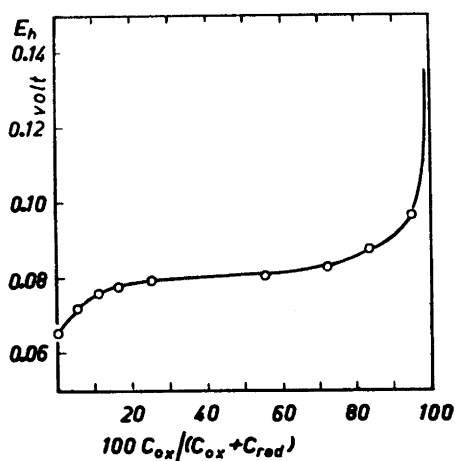


Fig. 2. Titration curve for *Ascophyllum* extracts.

RESULTS

Paper chromatography. Extracts from fresh and dried *Ascophyllum* were subjected to paper chromatography (S & S 2043 b). The solvent system used was butanol:pyridine:saturated sodium chloride solution, 1:1:2⁷. Bisdiazotised benzidine was used as spray reagent. The extracts from both fresh and dried weed showed two major spots with R_F values of approximately 0.18 and 0.95. In addition a weak spot with an R_F value of 0.70 and traces of other components were observed. The major spots observed using the diazo reagent could also be demonstrated by spraying with ammoniacal silver nitrate.

Potentiometric titration. In Fig. 1 the potential (E_h) of an extract of fresh *Ascophyllum* is plotted as a function of pH. It will be seen that the reducing power increases with increasing pH. The curve is of a complex shape, indicating a number of dissociation steps.

Experiments have been carried out with several oxidising agents, such as potassium ferricyanide, Fremy's salt, ceric ammonium sulfate, sodium nitroprusside and dichlorophenol-indophenol. Potassium ferricyanide in alkaline solution was found to be the most convenient oxidising agent and has been

Table 1. Reducing power, mequiv. per g dry matter.

Locality	Fresh	Dried
1. Tarva	2.25	2.20
2. Garten	1.56	1.70
3. Hysnes	2.25	2.20
4. Munkaunet	0.94	0.86
5. Korsvika	0.40	0.31
6. Leangen	0.67	0.73
7. Være	1.11	1.00
8. Midtsannan	1.27	1.46
9. Muruvik	0.63	0.67

used throughout this work. Satisfactory results were also obtained with Fremy's salt, while ceric ammonium sulfate, sodium nitroprusside and dichlorophenol-indophenol gave no satisfactory endpoint.

Fig. 2 shows a titration curve of an *Ascophyllum* extract. The index potential is low, indicating that a considerable number of electrons are involved in the reaction⁸.

Ascophyllum nodosum was collected at nine different localities in the Trondheimsfjord area in the first week of July 1957. The reducing power of fresh and dried material was determined. The results (Table 1) are given as milliequiv. per g dry matter.

It will be seen that there is no systematic difference between the reducing power of fresh and dried weed. The differences observed are probably due to incomplete extraction. Dried samples showed no loss of reducing power after three months of storage.

According to Rønnerstrand the vitamin C content of *Ascophyllum* corresponds to less than 0.02 mequiv. per g dry matter. Vitamin C makes therefore only a very small contribution to the reducing power of the extracts.

The reducing power of the *Ascophyllum* extracts varied from 0.4 to 2.25 mequiv. per g dry alga between the different localities. Localities Nos. 1, 2 and 3 are outside or in the outer parts of the Trondheimsfjord, localities Nos. 4, 5 and 6 are in the immediate neighbourhood of Trondheim, and localities Nos. 7, 8 and 9 are east of Trondheim, towards the inner parts of the fjord. Low reducing power was observed at localities where the salinity was lowered due to contamination with fresh water from river outlets. A more detailed study of these variations with place of growth will be published later.

Color reactions. In order to determine if the reducing compounds were identical with the substances responsible for the color reactions of the physodes, the nine *Ascophyllum* samples were extracted with dilute acid and the extracts subjected to color tests. As we wanted to include in the series an extract with a very low reducing power, a tenth sample was prepared in the following way: Water was added to the ground and dried material from locality No. 1 to reduce the dry matter content to 60%. The sample was then heated to 120°C for 30 min. After this treatment the reducing power of the sample had decreased to 0.05 mequiv. per g dry matter. A detailed study of the

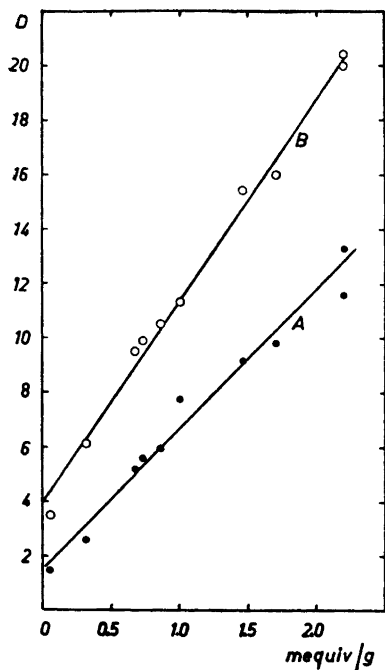


Fig. 3. Correlation between reducing power (mequiv. per g) and color with vanillin sulfuric acid (A) and diazosulfanilic acid (B) (optical density corrected for dilution).

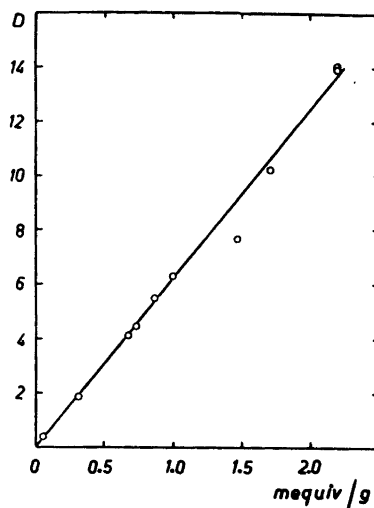


Fig. 4. Correlation between reducing power (mequiv. per g) and color of extracts after heating for 15 min at 120°C in alkali (optical density corrected for dilution).

effect of heat on the reducing power of the extracts will be published later.

In Fig. 3, curve A, the intensity of the red color of the extracts after addition of vanillin sulfuric acid is plotted as a function of the reducing power. The distinct correlation between the amount of reducing compounds and the red color given with vanillin sulfuric acid indicates that the substance in the physodes responsible for this reaction is identical with the reducing compounds.

Curve B in Fig. 3 shows the relation between the reducing power of the extracts and the color produced by diazotised sulfanilic acid in alkaline solution. Also in this case the correlation is pronounced and indicates that the reducing compounds and the compounds colored by diazo reagent are identical.

Fig. 4 shows the relation between the reducing power and the color obtained by heating the extracts for 15 min at 120°C after addition of alkali. The curve indicates that the reducing compounds are responsible for the formation of brown-colored substances in alkaline seaweed extracts.

It will be seen that the curve in Fig. 4 passes through the origin, while the curves in Fig. 3 pass through a point on the ordinate axis. This may be explained by assuming that the seaweed extracts contained a small amount

of a non-reducing substance that gave color reactions with diazotised sulfanilic acid and vanillin sulfuric acid, but which did not form brown colored substances when heated in alkaline solution.

The reaction of formaldehyde, gelatin and hide powder on the reducing compounds. When fresh *Ascophyllum* is treated with formaldehyde, the physodes become resistant to drying, freezing and boiling, while such treatment otherwise completely destroys the structure of the physodes. If an *Ascophyllum* sample treated with formaldehyde was extracted in the usual manner after careful removal of the formaldehyde *in vacuo*, the extract showed no reducing power, was not colored by the addition of diazo reagents or vanillin sulfuric acid, and furthermore no brown color was formed when the extract was heated after addition of alkali.

It is known that tannins are precipitated by gelatin. When a solution of gelatin was added to an *Ascophyllum* extract adjusted to pH 3, a precipitate was formed. When the precipitate was removed by centrifugation, the supernatant became only slightly colored on adding vanillin sulfuric acid or diazo reagent, or when the extract was heated with alkali. It could also be shown that only traces of reducing compounds were present.

Similar results were obtained when we used hide powder instead of gelatin as the protein component of the reaction. It was then possible to carry out the reaction quantitatively by determining the amount of dry matter removed from the extracts by the hide powder treatment⁹. For practical reasons, the extraction was in this case carried out with 40% (v/v) aqueous acetone solution. It was shown that this solvent extracted approximately the same amount of reducing compounds as the dilute acid used in the other determinations. The results are presented in Fig. 5 as a function of the reducing power of the acetone extracts. The curve can be extrapolated to a point on the ordinate axis corresponding to 4 g "tannins" per 100 g dry alga, indicating that the hide powder also reacted with non-reducing compounds in the extracts.

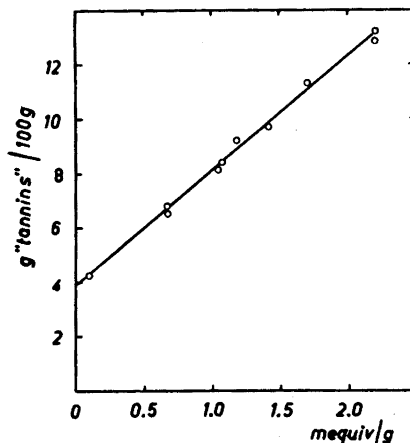


Fig. 5. Correlation between reducing power (mequiv. per g) and organic matter removed by hide powder treatment (g per 100 g dry matter).

The nature of these compounds is not known. If we assume that the content of interfering, non-reducing compounds is approximately the same in all samples, independent of the place of growth, the samples with the highest reducing power contain approximately 9 g reducing compounds per 100 g dry matter, which is in agreement with the result obtained when an equivalent weight of 40 is postulated.

DISCUSSION AND CONCLUSIONS

Extracts of *Ascophyllum nodosum* contain compounds which show a reducing potential measured against a saturated calomel electrode at pH values above 7. The relative amounts of these compounds can be estimated by potentiometric titration with potassium ferricyanide in alkaline solution. Plants from the outer parts of the Trondheimsfjord were found to contain about six times as much reducing compounds as a sample from a locality in the neighbourhood of Trondheim. Even the lowest reducing power observed corresponds to about ten times the "fucosan" content reported by Rønnerstrand. This is probably due to the fact that Rønnerstrand carried out his titrations in an acid medium, where the difference in potential between the seaweed extracts and the oxidising agent is small and a clear endpoint could not be obtained.

The pronounced correlation between the reducing power and the color reactions characteristic of the physodes strongly indicates that the reducing compounds are located in the physodes. This assumption is also supported by the fact that formaldehyde leads to a structural change in the physodes with the result that extracts of material treated with formaldehyde show no reducing power. Unpublished work by E. Baardseth of this institute indicated that the number of physodes per g fresh alga is positively correlated to the amount of reducing compounds in the plants.

The reactions of the reducing compounds with formaldehyde, gelatin, hide powder and color reagents are reactions characteristic of phenols. It is therefore probable that the physodes of *Ascophyllum* contain reducing, phenolic compounds. The results from the potentiometric titration and the analyses with hide powder showed that the reducing compounds occurred in considerable quantities, amounting to about 9 g per 100 g dry matter in the samples from the outer parts of the Trondheimsfjord.

So far the exact chemical nature of these compounds is not known. The results of this investigation indicate that the equivalent weight of the compound is low, that a number of electrons are involved in the reduction (Fig. 2) and furthermore that dissociation of the reducing groups occurs in several steps as the pH is increased.

REFERENCES

1. Crato, E. *Bot. Zeit.* **51** (1893) 157.
2. Kylin, H. *Hoppe-Seylers Z. physiol. Chem.* **83** (1913) 171.
3. Kylin, H. *Kungl. Fysiograf. Sällskap. Lund., Förh.* **8** [20] (1938) 1.
4. Jensen, A. og Haug, A. *Tidsskr. Kjemi, Bergvesen Met.* **8** (1952) 138.

5. Rønnerstrand, S. *Untersuchungen über Oxydase, Peroxydase und Ascorbinsäure in einigen Meeresalgen*. C. W. Lindström, Lund 1943.
6. Esping, U. *Arkiv Kemi* 11 (1957) 107.
7. Evans, R. A., Evans, W. C. and Parr, W. H. *Nature* 164 (1949) 674.
8. Jørgensen, H. *Redox-Maalinger*. Jul. Gjellerup, København 1945.
9. Friedrich, H. *Pharmazie* 9 (1954) 138.

Received February 7, 1958.