

Bacterial Growth Factors Related to Vitamin B₁₂ and Folic Acid in some Brown and Red Seaweeds

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The biological value of the seaweeds to some extent used as food and feed is scarcely explicable on the basis of analytical data concerning the normal major constituents. Recently the amino acids of some brown seaweeds have been investigated in this laboratory¹. The present experiments with seaweed extracts using different Lactobacilli as test organisms for microbiological growth factors, have demonstrated the natural occurrence of several water-soluble substances related to the animal protein factors and hematopoietic factors identified as vitamin B₁₂, folic acid (citrovorum factor, CF) and folic acid (FA).

The seaweeds studied in the present investigation belong to the following two classes of algae, viz., the *Phaeophyceae* and the *Rhodophyceae*. Three species from each class have been investigated, — from the *Phaeophyceae*: *Sphacelaria arctica* (of the order *Sphacelariales*), *Laminaria saccharina* (*Laminariales*), *Fucus vesiculosus* (*Fucales*), from the *Rhodophyceae*: *Furcellaria fastigiata* (of the order *Gigartinales*), *Polysiphonia nigrescens* and *Rhodomela subfusca* (*Ceramiales*). *Laminaria saccharina* was harvested on the west coast of Sweden near Fiskebäckskil (Skager Rack) during November. *Sphacelaria arctica*, *Fucus vesiculosus*, *Rhodomela subfusca* and *Furcellaria fastigiata* were collected at Simpnäs in the Åland Sea (the Baltic) in October, and *Polysiphonia nigrescens* was collected south of Bullerö in the Stockholm archipelago (the Baltic) in November 1951.

The algae were carefully cleaned and dried at room temperature as soon as possible after harvesting. 2–5 g of the dried samples were ground and extracted with 100 ml boiling water for thirty minutes to release the growth factors. The solutions were filtered and evaporated on a waterbath to 25 ml, filtered again, if necessary, and divided into two parts. One portion was autoclaved in 0.2 N. NaOH for 15 minutes at 120° C to destroy

any vitamin B₁₂, possibly present. Any residual activity on *Lb leichmannii* and *Lb lactis* Dorner must then be due to compounds other than vitamin B₁₂, such as desoxyribosides^{2,3}. These two solutions from each algal sample were then used for the estimation of vitamin B₁₂ and other growth factors.

Lactic acid bacteria utilised in the agar cup plate method have been used as test organisms in the following order: *Lactobacillus leichmannii* 313 (ATCC 7 830), *Lactobacillus lactis* Dorner 10 697, and *Lactobacillus lactis* Dorner 8 000. In this way a three-fold assay of vitamin B₁₂ type of activity was secured. *Leuconostoc citrovorum* 8 081 and *Streptococcus faecalis* 8 043 served for the folic acid (CF) and folic acid (FA) test respectively.

The agar cup plate method previously described⁴ was used for the different estimations with the first four Lactobacilli, and the method of Capps, Hobbs and Fox⁵, modified for the cup plate technique by replacing the sodium acetate by sodium citrate and incorporating 1.6 per cent of bacteriological agar in the single strength medium, was used for the *S. faecalis* test.

Crystalline vitamin B₁₂ (Cobemin, Merck), a synthetic citrovorum factor (folic acid, Leucovorin⁶, kindly supplied by Lederle lab. through the courtesy of Chemical Interests Co., Stockholm) and sodium salt of folic acid (Folivite Solution, Lederle), were employed as standards. Known amounts of vitamin B₁₂ were added to algal extracts in which the vitamin had been destroyed by autoclaving with alkali as described above. Three different concentrations of vitamin B₁₂ in such algal extracts covering a satisfactory assay range (*i. e.* standards with both higher and lower concentration of vitamin B₁₂ than that of the algal extracts not treated with alkali) were normally used and a dose-response curve was plotted. The exhibition rings of the test materials were then evaluated with the help of this curve. The results of vitamin B₁₂ estimations with three strains of Lactobacilli are shown in Table 1. The results in each column are mean values obtained from 5 to 30 single estimations. Results difficult to evaluate are represented by plus signs.

All the values in Table 1 are calculated in micrograms of vitamin B₁₂ standard per gram dry weight of the plant material. It is necessary to consider not only the values of the samples not treated with alkali but also the values of the alkali-treated samples to get a somewhat closer approach to the "true" vitamin B₁₂ contents. A summary of the approximate vitamin B₁₂ values is presented in the first column of Table 2. By calculating the figures in Table 2 the weighted mean values of Table 1 have been used. The second column of Table 2 gives the values of the apparent desoxyriboside contents expressed in vitamin B₁₂. It should be realized that the values are not directly proportional to the real quantities of desoxyribosides because the desoxyribosides promote the same growth for these Lactobacilli only at about four hundred times greater concentration than that of vitamin B₁₂. Columns 3 and 4 show the approximate amounts of citrovorum factor (CF) and folic acid (FA) activity liberated by the extractions.

The results obtained with these Lactobacilli in general use for the assay of vitamin B₁₂ evidently show the presence of vitamin B₁₂ in at least

Table 1.

Seaweed	Activity expressed as $\mu\text{g B}_{12}$ per g dry weight tested with					
	<i>Lb leichmannii</i> 313		<i>Lb lactis</i> D 10697		<i>Lb lactis</i> D 800	
	total	alkali-treated	total	alkali-treated	total	alkali-treated
<i>Sphacelaria a.</i> (number of determ.)	+	+	+	+	0.3	0.3
	(5)	(5)	(5)	(5)	(10)	(10)
<i>Laminaria s.</i> (number of determ.)	1	0.5	+	+	2 ^{a)}	1
	(15)	(10)	(5)	(5)	(15)	(8)
<i>Fucus v.</i> (number of determ.)	b)	b)	b)	b)	+	+
					(5)	(5)
<i>Furcellaria f.</i> (number of determ.)	1	0.5	0.5	+	+	1
	(20)	(15)	(15)	(15)	(10)	(10)
<i>Polysiphonia n.</i> (number of determ.)	1 ^{c)}	+	+	+	1 ^{c)}	+
	(10)	(5)	(5)	(5)	(30)	(15)
<i>Rhodomela s.</i> (number of determ.)	2	+	1	+	1	+
	(15)	(5)	(20)	(8)	(20)	(15)

a) double zones indicating the presence of at least two substances possessing bacteriological activity,

b) rings of successive salt diffusion in the agar gel covering the rings of bacterial growth,

c) inhibition rings, due to bacteriostatic substances.

three of the six seaweeds investigated, at the concentration of 0.5–1 μg per g dried plant substance *i.e.* 0.5–1 parts per million of vitamin B_{12} . In general, animal organs and non-photosynthetic microorganisms are considered as sources for vitamin B_{12} , and only traces of this type of growth factor are found in higher plants^{7,8}. Alfalfa has perhaps been investigated most thoroughly, but although it contains only 50 to 62 parts per billion of *Lb leichmannii* activity more than 85 % of this was found to be caused, not by vitamin B_{12} , but by other microbiologically active substances⁹. The level of vitamin B_{12} in these *Phaeophyceae* and *Rhodophyceae* is remarkably high.

Table 2.

Seaweed	Activity expressed as μg per g dry weight			
	Approximate values obtained from Table 1		<i>Leuconostoc citrovorum</i> 8 081	<i>Streptococcus faecalis</i> 8 043
	"true" vitamin B ₁₂ amounts	desoxyribosides expressed in B ₁₂	activity compared with Leucovorin standard CF	activity compared with Folivite standard FA
	1	2	3	4
<i>Sphacelaria a.</i> (number of determ.)	no	0.3	0.5 (15)	0.6 (5)
<i>Laminaria s.</i> (number of determ.)	0.5 ^{b)}	0.5	0.3 (8)	0.2 (5)
<i>Fucus v.</i> (number of determ.)	no	traces	0.2 (2)	0.2 (2)
<i>Furcellaria f.</i> (number of determ.)	c)	0.5	0.2 ^{a)} (15)	0.2 (5)
<i>Polysiphonia n.</i> (number of determ.)	1	traces	0.2 (12)	0.5 (5)
<i>Rhodomela s.</i> (number of determ.)	1	traces	0.2 (15)	0.3 (5)

- a) double zones indicating the presence of at least two substances possessing bacteriological activity,
 b) due to the disturbing influence of desoxyribosides (column 2) this figure is very uncertain. A double growth zone indicates the presence of vitamin B₁₂,
 c) due to the disturbing influence of desoxyribosides (column 2) no estimation of the vitamin B₁₂ content could be done. No double growth zones observed.

In *Fucus vesiculosus* and *Sphacelaria arctica* the concentration of vitamin B₁₂ may be too low for direct estimation by the cup plate method.

The occurrence of FA and CF was more expected in this kind of plant material than that of vitamin B₁₂. It should be pointed out that the growth of *L. citrovorum* can also be accounted for by thymidine (thymine desoxyriboside), but not by other desoxyribosides¹⁰.

The corresponding values of columns 3 and 4 show the existence of CF or high levels of thymidine. In those cases where the desoxyriboside effect measured with *Lb lactis* Dorner after alkali treatment is low or lacking (column 2) the values in column 3 and 4 may be due mainly to CF. Values in column 4 higher than those in 3 as for example in the case of *Polysiphonia* indicate the presence of FA in this material¹¹ at least. It is known that thymidine has quantitatively similar growth promoting effect for both *L. citrovorum* and *S. faecalis* but that FA gives a response with *L. citrovorum* only in concentrations about a thousand times as high as with *S. faecalis* and therefore the results with *L. citrovorum* could not be caused by FA. It would be quite unexpected to find only CF and no FA because of the close relationship between these factors and because of the general occurrence of FA in plant materials. Both CF and FA have been reported to occur in fresh natural materials of higher plants as well as in liver¹².

When *Furcellaria* was tested with *L. citrovorum* a double zone of stimulation appeared (Table 2, column 3) indicating the presence of at least two different growth factors. One of these factors was destroyed by the treatment with alkali described above.

Discussing the results it can be stated that vitamin B₁₂ and desoxyribosides (sometimes in high concentrations) as well as folic acid and folinic acid occur in certain brown and red algae. The inhibition of the growth of the lactobacilli caused by *Polysiphonia* as shown in Table 1 is of interest as it might be due to some kind of antibiotic.

SUMMARY

Aqueous extracts of three brown and three red marine algae have been investigated with respect to their contents of vitamin B₁₂, folinic acid and folic acid. Bioassays using three different Lactobacilli showed vitamin B₁₂ to be present in one brown, *Laminaria saccharina*, and two red algae, *Polysiphonia nigrescens* and *Rhodomela subfusca*, in a concentration of 0.5–1.0 µg per gram dry weight (due consideration having been taken to the influence of desoxyribosides). Folinic acid and folic acid were also detected.

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