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Bioautographic Separation of Vitamin B_{12} and Various Forms of Folinic Acid Occurring in Some Brown and Red Seaweeds

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The growth promoting activity of extracts of six different species of seaweed for *Lactobacillus leichmannii* 313 (ATCC 7 830), *Lb. lactis* Dorner (10 697), *Lb. lactis* Dorner (8 000), *Leuconostoc citrovorum* (8 081), and *Streptococcus faecalis* (8 043) was reported in a previous paper [1].

The present paper deals with chromatographic separation and bioautographic identification of the vitamin B_{12} and folinic acid (citrovorum factor, CF) type of growth factors in extracts of these algae.

EXPERIMENTAL

Materials and methods. Samples of *Phaeophyceae, i.e. Sphacelaria arctica (Sphacelariales)*, *Laminaria saccharina (Laminariales)* and *Fucus vesiculosus (Fucales)*, and of *Rhodophyceae, i.e. Furcellaria fastigiata (Gigartinales)*, *Polysiphonia nigrescens (Ceramiales)*, and *Rhodomela subfusca (Ceramiales)*, were collected on the east and west coasts of Sweden in the autumn of 1951, as reported previously [1].

Fresh algal samples were dried at room temperature, then ground and extracted with boiling water for 30 min. under reflux to release the growth factors. Drops of the extracts and of a standard solution were placed on 16 × 48 cm sheets of Whatman No. 1 filter paper and dried. These large sheets made it possible to run a standard solution and 4–5 extracts simultaneously. Descending chromatograms with *n*-butanol, water and acetic acid (125 : 125 : 30) were run overnight and the solvent front was allowed to advance about 40–42 cm from the starting line. The chromatograms were then dried.

Lactobacillus lactis Dorner (ATCC 8 000) and *Leuconostoc citrovorum* (ATCC 8 081) served as test organisms. The general technique associated with the maintenance of the

lactobacilli, the substrates etc. was the same as employed when using the agar cup plate method [2]. Tomato juice, fumaric acid and sodium-ethyloxalacetate were omitted from the assay medium in the case of *L. citrovorum*. Sterilized portions of the agar medium (150 ml) were cooled to 45—50°, mixed with 6 ml of a suspension of twice washed cells of *Lb. lactis* Dorner (LLD) (density: galvanometer reading 40 in Coleman spectrophotometer at 5 000 Å) or *L. citrovorum* (density: galv. reading 70) and poured into rectangular dishes of the same size as the chromatographic sheets. After solidification the chromatograms were spread out on the surface of the agar plates. The sheets were left on the agar plates during the whole incubation period (16—18 hrs) and removed only before readings were made. The incubation temperature was 37°. The development of *L. citrovorum* plates was found to be satisfactory at this temperature, which is higher than that recommended for the growth of this organism.

A solution containing 0.5 μg/ml of vitamin B_{12} (Cobemin, Merck), 0.1 mg/ml of guanine desoxyriboside (or alternatively hypoxanthine desoxyriboside), and 0.1 mg/ml of thymidine was used as a standard in the tests with LLD. The standard mixture used for the tests with *L. citrovorum* contained 0.1 mg/ml thymidine and 0.3 μg/ml of synthetic citrovorum factor (Leucovorin, Lederle, lot No. 7—1 142—4 B). A calcium salt of a natural citrovorum factor (Ca CF)-isolated from horse liver by Keresztesy and Silverman [3], and an injectable liver concentrate (Reticulogen, Lilly) was also used for comparison.

Separation of the LLD active factors in algal extracts. Crystalline vitamin B_{12} separates — if not protected from light — into two fractions, a slower fraction considered to be vitamin B_{12b} (hydroxocobalamin), and a faster moving fraction which is vitamin B_{12} (cyanocobalamin) [4,5]. The conversion of cyanocobalamin to hydroxocobalamin by light has been observed by several authors (*cf.*[6]).

As shown in Fig. 1, the extracts of three of the six algae tested, *viz. Laminaria saccharina, Polysiphonia nigrescens, Rhodomela subfusca*, seem to contain vitamin B_{12b} but none of them vitamin B_{12}. With the agar cup plate method we have already shown the existence of LLD active factors in these algae [1]. However LLD responds not only to vitamin B_{12} and B_{12b} but also to desoxybriosides if present in relatively high concentrations. Besides vitamin B_{12b} several desoxyribosides have been detected in these algal extracts. Thymidine was clearly present in all seaweeds investigated except *Fucus vesiculosus*. Cytosine-, guanine-, and hypoxanthine-desoxyridosides have very similar R_F-values in butanol-acetic acid as shown by Smith and Cuthbertson [4]. By employing the method described by Carter [7] for the separation of desoxyribosides, using Na_2HPO_4 in amyl alcohol as solvent, it was found that the slowest moving desoxyriboside present in *Sphacelaria arctica, Laminaria saccharina* and *Furcellaria fastigiata* was in all three cases hypoxanthine desoxyriboside.

There was no opportunity of testing a pure sample of adenine desoxyriboside, but one of the samples of thymidine employed as a standard contained some adenine desoxyriboside as impurity. The latter substance has a characteristic R_F-value between those of hypoxanthine desoxyriboside and thymidine and it was found that *Sphacelaria arctica* and *Furcellaria fastigiata* contained adenine desoxyriboside in quantities sufficient for detection. Other desoxyribosides possibly present in the seaweed extracts investigated, but in very low concentrations — less than 10 μg/ml of extract — as well as vitamin B_{12} if present only in concentrations less than 0.01 μg/ml, could not be detected in the extracts because such low levels were outside the range of the estimations.

Growth factors for Leuconostoc citrovorum. The organism *Leuconostoc citrovorum* 8 081, used originally in the research of the anti pernicious anemia (APA) principle [8], gives response with the natural and the synthetic citrovorum factor (CF, folinic acid) and with thymidine if present in high concentrations but not with the other desoxyribosides or with vitamin B_{12} [9,10]. The "folinic acid" or "citrovorum factor" has been shown to be N_5-formyl-5,6,7,8-tetrahydro folic acid [11,12].

Extracts of the algae have been chromatographed and the chromatograms developed bioautographically with *L. citrovorum* according to Winsten and Eigen [13] (Table 1). All the chromatograms except that of *Fucus vesiculosus* showed a thymidine spot with the same R_F-value as a spot developed on chromatograms with *Lb. lactis* Dorner (*cf.* Fig. 1). In at least four of the six seaweeds a citrovorum factor with the same R_F-value as that of the synthetic

Table 1. Distribution of growth factors, active towards Leuconostoc citrovorum 8 081, in red and brown seaweed extracts when chromatographed in a n-butanol, water, acetic acid mixture.

Spots with the same R_F-value as that of:	Rhodophyceae			Phaeophyceae		
	Sphacelaria arctica	*Laminaria saccharina*	*Fucus vesiculosus*	*Furcellaria fastigiata*	*Polysiphonia nigrescens*	*Rhodomela subfusca*
Leucovorin and Ca-CF	+	+	+	+*	+	nil
Thymidine	+	+	nil	+	+	+

* And other slowly moving factors (see text).

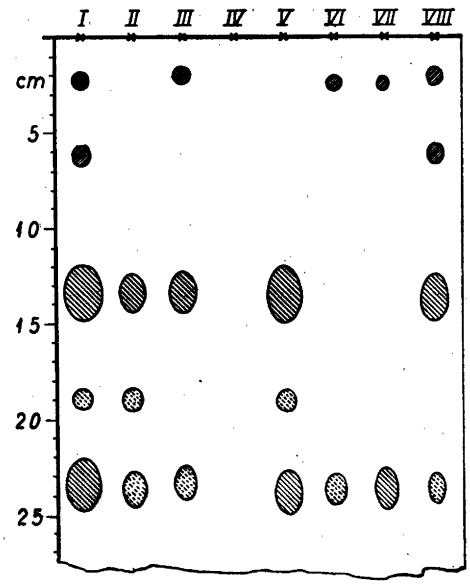

Fig. 1. Factors detected with Lactobacillus lactis Dorner 8 000. Columns I—VIII, spots representing zones of growth caused by:

I Standard: vit. B_{12b}, vit. B_{12}, desoxyribosides of hypoxanthine, adenine and thymine.

II Sphacelaria arctica: desoxyribosides of hypoxanthine, adenine and thymine.

III Laminaria saccharina: vit. B_{12b}, desoxyribosides of hypoxanthine and thymine.

IV Fucus vesiculosus: no factors for LLD.

V Furcellaria fastigiata: desoxyribosides of hypoxanthine, adenine and thymine.

VI Polysiphonia nigrescens: vit. B_{12b}, and thymine—desoxyriboside.

VII Rhodomela subfusca: vit. B_{12b}, and thymine—desoxyriboside.

VIII Reticulogen (Lilly): vit. B_{12b}, vit. B_{12}, desoxyribosides of hypoxanthine and thymine.

CF (tetrahydroformylfolic acid, Leucovorin) [12], was found. In our experiments the calcium salt of the natural CF (Ca CF) isolated from horse liver [14, 15] showed the same R_F-value as Leucovorin which is in agreement with the results reported by Sauberlich [16]. Dietrich *et al.* also found that autolyzed liver from several animal species gave spots similar to that of synthetic CF [17].

In addition to the CF detected in the algae, three further more slowly moving citrovorum factors were found in *Furcellaria fastigiata* as seen in Fig. 2, column II. The citrovorum factors of this alga were investigated more closely

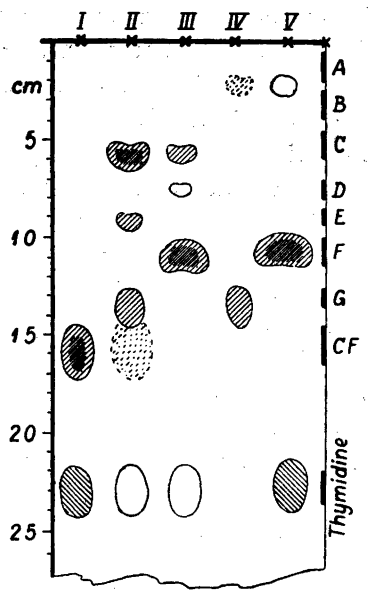

Fig. 2. Factors detected with Leuconostoc citrovorum 8 081. Solid lines indicate regular, dotted lines occasional growth. Shadowing was used when growth was more intensive. Columns I—V, spots representing zones of growth caused by;

I Standard: Leucovorin (Lederle) and Ca-citrovorum factor (in the same spot), and thymidine.

II Furcellaria fastigiata.

III Furcellaria fastigiata, enzyme treated.

IV Horse liver.

V Horse liver, enzyme treated.

as described below and were compared with liver and yeast which have been reported to possess CF of multiple nature [13].

Because synthetic di- and triglutamates of folinic acid are active towards *L. citrovorum* (*cf.* Shive *et al.*[11]) in a similar way, as di- and triglutamates of folic acid are towards *Lb. casei* and *S. faecalis* the existence of these conjugates of CF in nature was assumed. Winsten and Eigen [13] reported in their early work two slowly moving growth factors for *L. citrovorum* in liver which could be converted by an enzyme in rat stomach homogenate to a faster moving factor with high growth promoting activity. Furthermore Hill and Scott [18] have shown that dried brewers' yeast contains the CF largely in bound form, from which it can be released by a hog kidney extract known to contain folic acid conjugase. From the results obtained they conclude "that folic acid conjugase and the CF-liberating enzyme of hog kidney are strikingly

similar if not identical". For these reasons the hog kidney enzyme was used for the comparison of the citrovorum factors in *Furcellaria*, liver and yeast.

Chromatograms were made of portions of a hot water extract of *Furcellaria*, of fresh horse liver homogenated in a Waring blendor (1 g of fresh liver per 10 ml of water) and of a solution of yeast extract (Difco) steamed for 30 min. in an autoclave (1 g per 10 ml water). In order to release the citrovorum factors from their microbiologically inactive bound form or from their conjugates, similar to those of folic acid (*cf.* [16—19]), other portions of the same extracts were mixed with equal volumes of a suspension containing 1 g hog kidney homogenate and 0.14 mole cysteine HCl per liter, according to Hill and Scott [18, 19]. The suspension was adjusted to pH 4.5. The extracts were digested under toluene at 40° C for at least 16 hours. After evaporation of the toluene they were chromatographed and the chromatograms developed with *L. citrovorum*. The kidney extract did not contain significant amounts of factors stimulating the growth of this lactobacillus. Fig. 2 shows the results obtained with *Furcellaria* and liver.

Liver does not seem to contain a factor identical with the slowest moving factor in *Furcellaria*, neither before nor after enzyme treatment, although liver gives a spot representing a still lower R_F-value. Both *Furcellaria* and liver have a factor — on the horizontal zone *G* — with R_F-value slightly lower than that of the synthetic CF and Ca CF of horse liver (zone *CF*). The main effect following enzymatic digestion appears to be that in the case of both *Furcellaria* and liver a heavy spot due to a factor in the zone *F* appeared. The slow moving factor, zone *C*, in *Furcellaria* decreased on the same treatment.

Enzymatic digestion of yeast extract (Difco), released four factors with R_F-values lower than that of tetrahydroformylfolic acid. Yeast extract showed features different from those of liver and *Furcellaria* before and after digestion but it was not further investigated whether any of the factors of yeast extract were identical with the CF type of factors of extract of horse liver and the alga *Furcellaria*. Doctor and Couch [20] found, however, that the microbiologically inactive precursors of the CF in liver and in yeast are similar at least in the respect of their movement in different solvent systems. It is of interest to note that in our experiments the CF-conjugase of hog kidney did not liberate CF itself but instead only more slowly moving factors. The experiments clearly show that several naturally occurring factors active towards *L. citrovorum* exist, some or all of which may be conjugates of folinic acid (CF).

SUMMARY

Paper chromatograms of hot water extracts of three brown and three red seaweeds were developed bioautographically using *Lactobacillus lactis* Dorner, and *Leuconostoc citrovorum*.

1. Vitamin B_{12b} seems to be present in the following three algae: *Laminaria saccharina*, *Rhodomela subfusca* and *Polysiphonia nigrescens*.

2. The desoxyribosides of adenine, hypoxanthine and thymine seem to be present in different concentrations in the different algae.

3. In five algae, *Sphacelaria arctica*, *Laminaria saccharina*, *Fucus vesiculosus*, *Furcellaria fastigiata* and *Polysiphonia nigrescens*, a citrovorum factor similar to N_5-formyl-5,6,7,8-tetrahydrofolic acid was found. *Furcelleria fastigiata*, the alga richest in growth factors, contained three slow-moving citrovorum factors other than formyltetrahydrofolic acid. A comparison of the citrovorum factors of *Furcellaria fastigiata* and of horse liver before and after treatment with hog kidney homogenate, which contains CF-conjugase, shows that *Furcellaria* seems to contain two slow moving factors which are not present in horse liver. The same treatment released several slow moving citrovorum factors in a yeast extract (Bacto).

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