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On the Occurrence of Light-sensitive Corrinoids* in Axenic Cultures of Unicellular Algae

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1. Species of unicellular green (*Chlorella pyrenoidosa*), blue-green (*Anabaena cylindrica*), and red (*Rhodosorus marinus*) algae were grown in axenic cultures in the presence and in the absence of vitamin B_{12} or its analogues.

2. Extracts from such algae were studied with respect to the occurrence of light- and cyanide-sensitive derivatives of vitamin B_{12} by means of a bioautographic technique involving chromatography and/or ionophoresis.

3. Extracts from all the three species investigated contained several corrinoid compounds including at least one light-sensitive substance moving on chromatograms considerably faster than the "B_{12}-coenzyme" (DMBC).

4. DMBC was found along with the faster moving light-sensitive material in extracts from *Chlorella* and in extracts from *Rhodosorus* only when the algae were grown in the presence of cyanocobalamin, but not in the other cultures.

5. All three species were found to convert exogenously supplied cyanoforms of vitamin B_{12}, or certain other cobamides, to other derivatives, amongst which some at least were light-sensitive.

6. The proportion of the light-sensitive corrinoids was largest in the extracts from *Anabaena*, in certain cultures up to 70–80 % of the total *E. coli 113–3* activity.

7. No significant differences in the distribution of the different corrinoid spots were observed between *Anabaena* cultures grown in the presence or in the absence of a nitrogen source in the medium.

* The nomenclature system described by Smith [30] will be used for the cobalt-corrin compounds along with various trivial names that are currently employed in the B_{12}-field. B_{12} or cyanocobalamin refers to α-(5,6-dimethylbenzimidazolyl) cobamide cyanide. A derivative of B_{12} in which the cyanide is replaced by a hydroxy group is called aquocobalamin. The prefix "aquo" is also employed to describe the corresponding hydroxy derivatives of B_{12}-analogues. Factor III is the α-(5-hydroxybenzimidazolyl) cobamide cyanide; ψ-B_{12} means α-adenylcobamide cyanide; Factor A is α-(2-methyl-adenyl) cobamide cyanide; Factor B is cobinamide.

Whereas the possible participation of vitamin B_{12} in the metabolism of higher plants is still a matter of controversy [1] it was realized very soon after the isolation of this vitamin in 1948 that it participated in the metabolism of certain unicellular algae. Several species of these organisms are heterotrophs with respect to vitamin B_{12} or its analogues.[2] Brown, Cuthbertson and Fogg have already shown as early as 1956 that the unicellular blue-green alga *Anabaena cylindrica* and the green alga *Chlorella vulgaris* produced considerable amounts of compounds with vitamin B_{12} activity.[3] Fries has demonstrated that vitamin B_{12} also participates in the metabolism of marine red algae.[4] The latter author has isolated two multicellular algae, *Goniotrichum elegans* [5] and *Polysiphonia urceolata* [6] both of which are absolute heterotrophs with respect to vitamin B_{12}. The vitamin B_{12} requirement of these organisms can be satisfied not only with cyanocobalamin, but also, to a varying extent, with a number of B_{12} analogues. It seems interesting in this connection that one of these active analogues, *viz.* Factor Z1 isolated from sewage,[7] is a compound which has practically no growth stimulating activity on the conventional test organisms such as *O. malhamensis*, *L. leichmannii* or even the very unspecific *E. coli 113—3*.

It is now well established that vitamin B_{12} and its analogues do not function directly as catalysts in enzymatic reactions, but must be converted first into more complex coenzyme forms.[8,9] The naturally occurring corrinoid compounds possessing coenzyme activity that have been so far characterised are the 5′-deoxyadenosylcobamides.[10] These coenzymes have been isolated from bacteria and animals. No one has hitherto reported experiments to assay algae for their occurrence. It was felt that a demonstration of the occurrence of cobamide coenzymes in unicellular algae grown in axenic cultures would give support to the view that vitamin B_{12} participates in certain vital enzymatic reactions in such organisms and does not merely accumulate in the cells as a result of some unspecific processes.

The 5′-deoxyadenosylcobamides are extremely light-sensitive and are also sensitive to acid and cyanide treatment. Three enzymatic reactions, controlled by the concentration of these coenzymes, have been hitherto elucidated.[11] The requirement for these coenzymes has also been demonstrated in certain other *in vitro* reactions. However, the enzyme assays available are not applicable to materials with comparatively low B_{12} content (*cf.* Ref. 12)which is the case with algae. Furthermore, it is uncertain whether the nucleotide lacking B_{12} analogues which seem to be of interest with respect to certain red algae [5,6] can form coenzymes which are active in the available enzymic assays or not. Other nucleotide lacking coenzymes isolated from bacteria have been shown to be inactive in the glutamate mutase assay.[11]

Although methods are described in the literature for the isolation and identification of B_{12} coenzymes by spectrophotometric and chemical means, such methods require comparatively large amounts of B_{12} material. The vitamin B_{12} content of the algae studied was of the order of magnitude 0.1—1.0 μg/g fresh algae. It would have been necessary to extract kilograms of algae in order to obtain solutions of the individual B_{12} derivatives, suitable for spectrophotometric analysis. Algae in axenic cultures grow rather poorly. The yields which can be obtained — even on a comparatively large

laboratory scale — amount to gram quantities, at the most. We had therefore to select a sensitive detection method as the first approach to the problem of a possible occurrence of cobamide coenzymes in algae.

Volcani *et al.* have applied the ionophoretic-bioautographic method of detecting cobinamide and cobamide vitamins in millimicrogram quantities to the detection of the 5'-deoxyadenosylcoenzymes in microbial extracts.[12] A corresponding chromatographic-bioautographic method has been found in this laboratory to be even more useful for the study of cobamide coenzymes in algal extracts, especially when using the solvent employed by Pawelkiewicz *et al.*[13] and by Zagalak and Pawelkiewicz.[14] In addition, a procedure has been introduced consisting of simultaneous chromatography of algal extracts prepared in different ways.

EXPERIMENTAL PROCEDURE

Algae

Rhodosorus marinus, *Anabaena cylindrica* and *Chlorella pyrenoidosa* were selected as representatives of the red, blue-green, and green algae, respectively. They are all unicellular species familiar from previous plant physiological investigations. All three species are vitamin autotrophs, and members of the genera *Anabaena and Chlorella* are known to be corrinoid producers.[3] As all the hitherto investigated multicellular red algae have turned out to be more or less dependent on an exogenous source of corrinoids it was presumed that these substances may also participate in the metabolism of *Rhodosorus*. *Anabaena cylindrica* and *Chlorella pyrenoidosa* have been obtained in axenic cultures from the Collection of Algae and Protozoa, Botany School, Cambridge, England. *Rhodosorus marinus* was gifted in unialgal culture by Dr. G. Giraud, École Normale Supérieure, Paris, France, and has been obtained free from bacteria after treatments with antibiotics.[15]

Cultivation, harvest and storage of algal cells

All three algal species were cultivated under rigorously aseptic conditions in completely defined mineral salt solutions and carefully tested so that they were free from all contaminants. All chemicals used were of commercially available reagent grade and redistilled water was used throughout the investigation.

The cultures were illuminated for 18 h by fluorescent tubes giving approximately 2000 Lux, at 20°C.

Rhodosorus marinus was cultivated in artificial sea water ASP 6F according to Fries.[15] In some experiments cyanocobalamin or Factor III was added whereas all other vitamins were omitted. Incubation time was 20 days. Methods of cultivation and harvesting were as described earlier.[15]

Chlorella pyrenoidosa was cultivated in a mineral salt solution according to Lindahl,[16] usually in 100 ml Erlenmeyer flasks containing 25 ml of the nutrient medium. Each sample investigated therefore represented a batch collected from 15—40 flasks. Growth was considerably increased when the nutrient medium contained 0.1 % of glucose and the algae were cultivated in 1000 ml Fernbach flasks containing 250 ml medium through which sterile air was bubbling. Incubation time was 14 days.

Anabaena cylindrica was grown in a mineral salt medium according to Fogg,[17] but with ferric citrate instead of ferric chloride. Other conditions of cultivation were similar to those employed for *Chlorella*. Aeration in Fernbach flasks increased the growth by 100 %, but was without any observable effect on the corrinoids detected. Since it has been suggested recently that vitamin B_{12} may be involved in the nitrogen fixation process,[1] certain experiments were conducted under the conditions of nitrogen fixation or using different nitrogen sources in the growth medium. The growth without a nitrogen source in the medium was comparable to that in the presence of sodium nitrate at a concentration

of 200 mg/l. Arginine, at equivalent nitrogen levels, increased the growth by 100 %. When nitrogen was supplied as an ammonium salt or as asparagine, at different concentration levels, the growth was very poor. Incubation time was 20 days.

On harvesting, the contents of the flasks were centrifuged and the cells were further separated from the nutrient medium by filtration on a suction funnel, followed by rapid washing with distilled water. The cells were then immediately frozen. Certain batches were freeze-dried. All material was stored at −20°C until used.

Extraction and enrichment of the corrinoids

Aliquot portions of the algal cell material were, in most experiments, extracted by two different methods, *viz.*

A. under conditions favouring stability of the coenzymes, *i.e.* at neutral pH, in the absence of CN^- and in the dark.

B. under conditions favouring the conversion of the corrinoids to their cyano derivatives, *i.e.* in the presence of 0.01 M KCN.

Aliquot portions of the extracts obtained according to method A were illuminated with a 75 W incandescent lamp for 1−3 h. The solutions, in approximately 1 cm thick layers in small beakers, were placed 30 cm below the lamp in a box lined with aluminum foil in order to secure uniform illumination of the samples.

Using approximately 1 g of frozen or 200 mg of freeze dried cells per 50 ml of the extracting medium several extraction procedures were investigated, *e.g.* extraction with either acetate or phosphate buffer; with methyl, ethyl or isopropyl alcohol; heating in a boiling water bath as opposed to autoclaving at 121°C; grinding with glass powder or dry ice, and disintegration of the cells in a French press. The procedures finally adopted in most experiments reported here involved heating in a boiling water bath for 15 min using one of the following solutions: 1. Sodium acetate buffer, 0.01 M, pH 6.0; 2. Similar to 1, but containing 0.01 M KCN; 3. 80 % ethyl alcohol. The extraction was carried out in centrifuge tubes covered with aluminum foil. The heating was followed by an immediate cooling in running tap water and centrifugation at approx. 3000 *g* at ambient temperature. The supernatant was removed, the residue was washed once with cold extraction medium and the washing was combined with the extract. More extensive washing was found to be superfluous. The water extracts were then immediately frozen. The ethanol extracts were freed from alcohol *in vacuo* at 40°−50°C (usually overnight) and the water residues were freeze dried. The dry residues were taken up in small volumes of sodium acetate buffer, 0.01 M, pH 6.0, to give a concentration of B_{12} activity corresponding to 0.05−0.1 μg/ml as determined by *E. coli 113−3 plate* assay. Approximately 10−30 μl of such solutions were applied to paper sheets for subsequent ionophoresis and/or chromatography. The extraction with 80 % ethanol gave the purest preparations and this permitted application of comparatively larger amounts of samples to the ionophoretograms and chromatograms than was possible with the buffer extracts. The ethanolic extracts, however, had to be freed from alcohol before they could be freeze dried. This necessitated a considerably extended time of evaporation in the liquid state with an inherent risk of the decomposition of the coenzymes. All operations were carried out either in the dark or in a very dim light.

Chromatography. Chromatography was carried out on Whatman 3 MM paper at 37°C, in the dark using the descending technique. Three solvent systems were employed, *viz.* I. *sec.* butanol, acetic acid, water: 100:1:50; II. *sec.* butanol, acetic acid, water: 100:1:50, containing 0.01 M KCN; III. butanol, propan-2-ol, acetic acid, water: 100:70:1:100 as described by Pawelkievicz *et al.*[13]

The solvent system III gave the most satisfactory separation. In the chromatography experiments only the solution prepared from the ethanol extracts were employed. These samples were always run simultaneously with aliquot portions of the same preparations which had previously been illuminated as described above or heated with cyanide. Authentic samples of DMBC, cyanocobalamin, aquocobalamin and/or certain other cobamides and cobinamides were also run alongside the algal extracts.

Ionophoresis. Paper No. 507 from Schleicher and Schüll as recommended by Volcani *et al.*[12] and a solvent consisting of 0.5 M acetic acid as described by Ford *et al.*[18] were used in all ionophoretic experiments. The LKB Paper Electrophoresis Apparatus Type 3276

B was employed and the separation was carried out for 18 h, at +4°C, in the dark, at 10 V/cm. Usually, the extracts obtained according to methods A and B above were subjected to ionophoresis simultaneously and alongside authentic specimens of the 5,6-dimethyl-benzimidazolyl-cobamide coenzyme (DMBC), cyanocobalamin and/or aquocobalamin. The ionophoretic experiments were carried out only with the red algae and were abandoned in studies with other species.

Bioautography. E. coli 113—3 was employed as the test organism and the simple mineral medium described by Diding [19] was used in most experiments. This medium was supplemented with thiomalic acid, 0.1 g/l and with KCN, 1 mg/l, and was autoclaved together with glucose. Large agar plates (18 × 36 cm) containing 150 ml of this medium were seeded with the test organism. The dried ionophoretograms or chromatograms were placed on the agar and the plates were incubated overnight at 37°C. In certain experiments Burkholders's medium [20] was used instead of the mineral medium. The spots obtained in the plate assay were then usually sharper and better separated, but the zones were much smaller. Certain smaller and/or weaker spots obtained with Diding's medium did not appear when Burkholder's medium was used.

It proved to be more satisfactory in certain cases, especially with ionophoretograms, to cut the paper in 1 cm sections and to locate the spots by means of turbidimetric assay.

Reference substances. Methyl-cobalamin decomposed rather rapidly in dilute solutions (0.05—0.1 μg/ml) whereas it was more stable in concentrated ones (1—10 mg/ml). Freshly prepared solutions of methyl-cobalamin and DMBC were used in certain crucial experiments. Otherwise, the solutions were divided in small portions and kept frozen at —20°C. Each portion was thawed only once. The chromatographic mobilities of methyl-cobalamin, DMBC as well as of B_{12} and aquocobalamin were determined using both, visible spots and bioautography. The two methods gave essentially similar results. Methionine was localized on the chromatograms by the ninhydrin reaction.

RESULTS

In Table 1 the results of studies on the chromatographic-bioautographic mobilities of corrinoids in three different solvents are summarized. They have been extracted from certain green, blue-green, and red algae grown in axenic cultures.

The mobilities are expressed as R_{DMBC}, *i.e.* in relation to the mobility of the 5′-deoxyadenosyl coenzyme of vitamin B_{12}. The extracts investigated were prepared under conditions favouring the stability of B_{12}-coenzymes. It can be seen that extracts of all the species investigated contain several corrinoid compounds including at least one light-sensitive derivative moving faster than DMBC. Solvent I gave essentially the same pattern as solvent III, but the movement of the corrinoids was much slower and the separation considerably poorer in the former solvent than in the latter one.

Chlorella. A representative chromatographic-bioautographic pattern of extracts from *Chlorella pyrenoidosa* using solvent III is shown in Fig. 1. In this case the alga was grown with 0.1 μg B_{12}/ml. It can be seen that at least two light-sensitive corrinoids, corresponding to spots a and c, respectively, are present in the extracts. Spot c may be tentatively identified as DMBC. Spot a, which is the predominating one, moves faster than vitamin B_{12}. Upon illumination it changes to a spot with the chromatographic mobility of aquocobalamin and is converted to vitamin B_{12} by cyanide treatment. Of the remaining two spots, spot b probably corresponds to the unchanged vitamin B_{12} whereas spot d probably represents a mixture of several compounds amongst which aquocobalamin is also present. This is indicated by the shape of spot d and its alteration upon illumination or cyanide treatment.

Table 1. Chromatographic mobilities of corrinoids extracted from green, blue-green and red unicellular algae under conditions favouring the stability of B_{12}-coenzymes (Method A). Details of procedure can be found in the text.

Species and conditions of growth	R_{DMBC} (±0.1) in solvent I	II	III
Chlorella pyrenoidosa grown with glucose			
without vitamins	0.9	1.2	0.9
	1.6[a]	1.4[a]	1.2[a]
with B_{12}			0.8
			1.0[a]
			1.2[d]
			1.5[a]
Rhodosorus marinus grown without vitamins			0.7
	0.3[b]		0.9
	0.7[b]		1.1[b,a]
	0.9[b]	0.9	1.3[b]
	1.5[a]	1.6[a]	1.5[a]
		2.1[a] trace	
Anabaena cylindrica grown without nitrogen			
without vitamins	0.4		0.4
	0.8	0.7	0.8
	1.3[a]	1.4	1.2[a]
	1.8[a,c] trace	2.2[a,c] trace	1.5[a,c] trace
Reference substances			
DMBC	1.0	1.0	1.0
Vitamin B_{12}	1.3	1.2	1.2
Aquocobalamin	0.7	1.2	0.7
Methyl-cobalamin	2.2	2.0	1.6
Methionine	5.2	1.9	1.5

[a] light sensitive.
[b] poor separation.
[c] only in certain cultures.
[d] partly light sensitive.

When *Chlorella pyrenoidosa* was grown without vitamins in the medium, only two spots, one of them light-sensitive, were detected in the extracts. In solvent III the light-sensitive spot was indistinguishable from vitamin B_{12} (*cf.* Table 1).

Rhodosorus marinus. Chromatographic-bioautographic patterns of extracts from *Rhodosorus marinus* grown without vitamins and with Factor III in the medium, are given in Figs. 2:1 and 2:2. Three main spots can be seen in Fig. 2:1, two of which, b—c and d—e, seem to be mixtures of at least two substances each. As in the case of *Chlorella* (*cf.* Fig. 1) there is a fast moving,

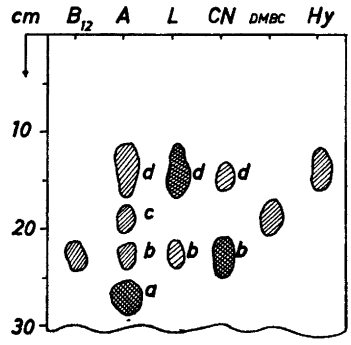

Fig. 1. Chromatographic-bioautographic patterns of extracts from *Chlorella pyrenoidosa* grown with 0.1 μg B_{12}/ml. Extraction with 80 % ethyl alcohol, 15 min at 100°C in the dark (A); extracts illuminated (L) or heated with 0.01 M KCN (CN). Solvent III, descending technique, 18 h at 37°C. Detected on agar plates seeded with *E. coli 113—3*. *a*, unidentified light-sensitive corrinoid; *b*, vit. B_{12}; *c*, DMBC; *d*, mixture containing aquocobalamin (Hy) as one of the components.

light-sensitive spot (spot a) with a similar chromatographic mobility. Only a trace of this spot remains after cyanide treatment along with the two main spots (b and d—e). These are difficult to identify as they may represent mixtures of several corrinoid compounds (*cf.* Table 3). When *Rhodosorus* was grown in the presence of Factor III or in the presence of cyanocobalamin

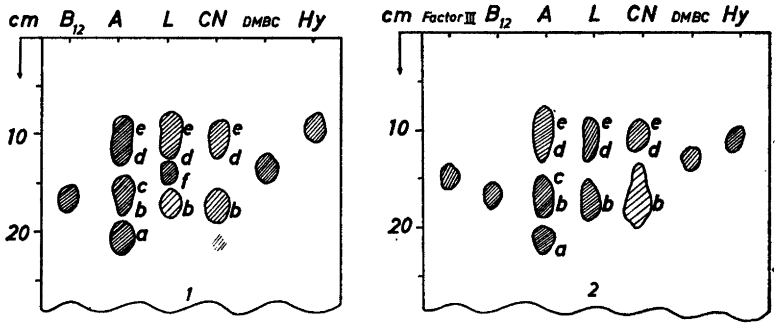

Fig. 2. Chromatographic-bioautographic patterns of extracts from *Rhodosorus marinus* 2:1 grown without vitamins; 2:2 grown with Factor III, 0.1 μg/ml. Details of the procedure and symbols as in legend to Fig. 1. *a*, unidentified light-sensitive corrinoid; *b-c*, mixture containing a light-sensitive corrinoid and probably Factor III and/or B_{12}; *d-e*, mixture containing aquoforms of B_{12} and/or Factor III as some of the components; *f*, unidentified.

(0.1 μg per ml medium in either case) the unidentified cyanide-sensitive spot f was absent (for Factor III, *cf.* Fig. 2:2). Extracts from cells grown in the presence of cyanocobalamin contained in addition to the fast-moving light-sensitive material (Figs. 2:1 and 2:2, spot a) also unchanged cyanocobalamin, aquocobalamin and a light-sensitive substance with a chromatographic mobility similar to that of DMBC.

Results of ionophoretic studies on *Rhodosorus* extracts are summarized in Fig. 3:1—3. Extracts of cells grown without vitamins (Fig. 3:1) give rise to five bands when prepared in the dark and in the absence of CN^-, but only one band when prepared in the presence of KCN. The nature of the respective light- and cyanide-sensitive bands is difficult to elucidate (*cf.* Discussion).

When the alga is grown with Factor III the pattern of the extract is quite different (Fig. 3:2) and it is different again when cyanocobalamin is present in the growth medium (Fig. 3:3). Tentative identification of some of the observed bands is given in the legend to Fig. 3. It appears from Fig. 3 that the cells of *Rhodosorus* have the ability to convert the cyano forms of Factor III and vitamin B_{12} to other compounds.

Anabaena cylindrica. The studies on chromatographic mobilities of corrinoids extracted from *Anabaena cylindrica* grown under different conditions are summarized in Table 2. Four main types of cultures were investigated, *viz.* with or without a nitrogen source in the medium and both of these with or without exogenous B_{12}. It can be seen in Table 2 that no striking differences were observed between the chromatographic patterns of the extracts from the four different cultures. All of them contained a light sensitive corrinoid, in mixtures indistinguishable from vitamin B_{12}. This material may correspond to the light sensitive derivative observed in extracts from *Chlorella* grown without vitamins (*cf.* Tables 1 and 2). This light-sensitive spot (R_{DMBC} = 1.2) constituted a large fraction of the total B_{12} activity of the *Anabaena* extracts, in certain cultures up to 70—80 %.

Table 2. Chromatographic mobilities of corrinoids extracted from *Anabaena cylindrica*. Details of the procedure and symbols as in the legend to Fig. 1.

Conditions of growth	No. of cultures investigated	Extracts according to R_{DMBC} (±0.1) A	L	CN
1. without nitrogen in medium	4	0.4	0.4	0.4
		0.8	0.8 domin	0.8
		1.2 domin	—	1.2 trace
		1.6 trace[a]	—	1.6 trace[a]
2. as 1 above, but with B_{12} at a concentration of 0.001 or 1 μg/ml	3	0.4	0.4	0.4
		0.8	0.8 domin	0.8
		1.2	—	1.2 trace
		1.6[b]	—	1.6 trace[b]
3. with nitrogen in medium				
a. nitrate	1	0.4 trace	0.4	0.4
		0.8	0.8	0.8
		1.2 domin	—	1.2 trace
b. arginine	2	0.4 trace	0.4 trace	0.4
		0.8	0.8	0.8
		1.2 domin	—	1.2 trace
4. as 3a, but with 1 mμg B_{12}/ml	1	0.4 trace	0.4	0.4
		0.8	0.8	0.8
		1.2	—	1.2 trace

[a] did not occur in all cultures.
[b] only with 1 μg B_{12}/ml.

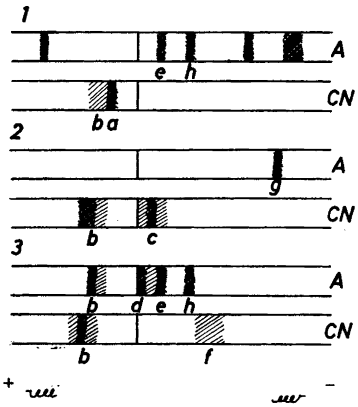

Fig. 3. Ionophoretic-bioautographic patterns of extracts from *Rhodosorus*. *1*, grown without vitamins; *2*, grown with Factor III 1 mμg/ml; *3*, grown with cyanocobalamin 1 mμg/ml. Ionophoresis in 0.5 M acetic acid on Schleicher and Schüll paper No. 507; 18 h at 10 V/cm and +4°C. Bands detected by the turbidimetric assay using *E. coli 113—3*. Extraction of algae with 0.01 M sodium acetate, pH 6.0 under conditions: A, known to favour the stability of B_{12}-coenzymes; CN, in the presence of 0.01 M KCN at 100°C. Tentative identification of bands by comparison with authentic specimens, chromatographic experiments (*cf.* Tables 1 and 3) and literature references: *a,b*, mixture possibly containing some or all of the following: B_{12}, ψ-B_{12}, Factor III, Factor A; *c,d*, unidentified; *e,g, h*, light-sensitive; *f*, cobinamide?

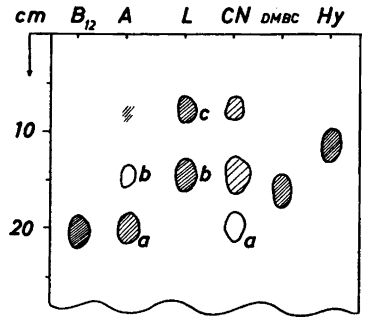

Fig. 4. Chromatographic-bioautographic patterns of extracts from *Anabaena cylindrica* grown without vitamins and without a nitrogen source in the medium. Details of the procedure and symbols as in legend to Fig. 1. *a*, light-sensitive corrinoid constituting a major part of the *E. coli* activity of the extracts; *b*, partly ψ-B_{12}, partly unidentified; *c*, probably a mixture containing aquo-derivative of ψ-B_{12} as one of the components.

The other light-sensitive corrinoid (R_{DMBC} = 1.5 in solvent III) detected in the green and red algae (*cf.* Table 1) was only occasionally found in *Anabaena* and exclusively in cultures grown without nitrogen in the medium (Table 2, R_{DMBC} = 1.6). The proportion of this compound was rather small.

A representative chromatographic-bioautographic pattern of extracts from *Anabaena*, grown without vitamins and without a nitrogen source in the medium, is given in Fig. 4. It can be seen that the predominant corrinoid in such extracts is a light-sensitive derivative (spot a) moving faster than DMBC, and, in mixtures, hardly distinguishable from vitamin B_{12}.

Experiments to characterize the fast-moving light-sensitive material. The fast-moving light-sensitive material found in extracts from *Chlorella* and *Rhodosorus* was studied further by means of co-chromatograms with solutions of crystalline methyl-cobalamin. The results are shown in Figs. 5:1 and 5:2. The fast-moving light-sensitive material from both *Chlorella* and *Rhodosorus* was indistinguishable from methyl cobalamin on the chromatograms run in solvent III (Fig.

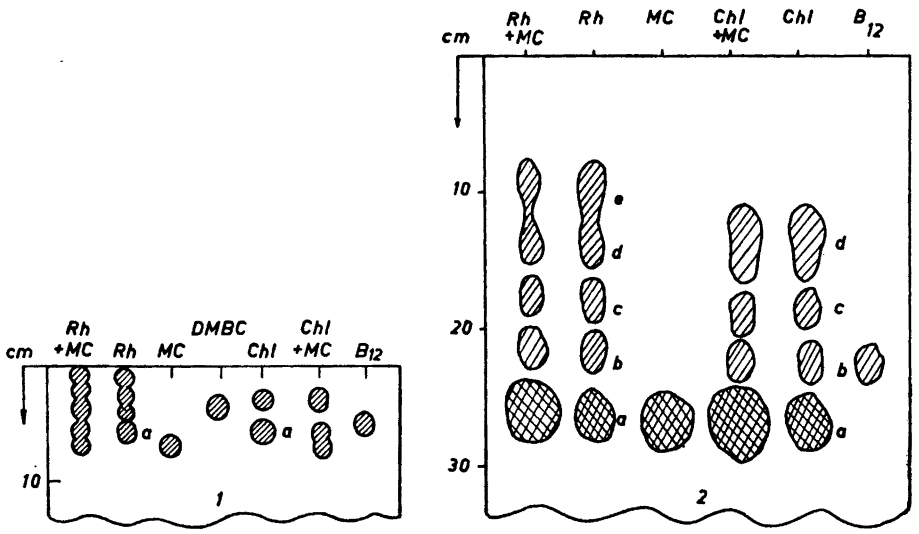

Fig. 5. Chromatographic-bioautographic patterns of extracts from *Rhodosorus* (Rh) and *Chlorella* (Chl) grown in the presence of vit. B_{12} (0.1 μg/ml). Details of the procedure (Method A) as in legend to Fig. 1. 5:1 Solvent I, 5:2 Solvent III, MC, methyl-cobalamin.

5:2). When solvent I was employed the position and the shape of the mixed spots indicated that the two materials are not identical, but a satisfactory separation to prove this point could not be achieved (*cf.* Fig. 5:1).

In another set of experiments algal extracts were applied as streaks to a number of Whatman 3MM sheets and subjected to chromatography in solvent III. Strips, 2 cm wide, were cut at the left and the right side of each sheet and

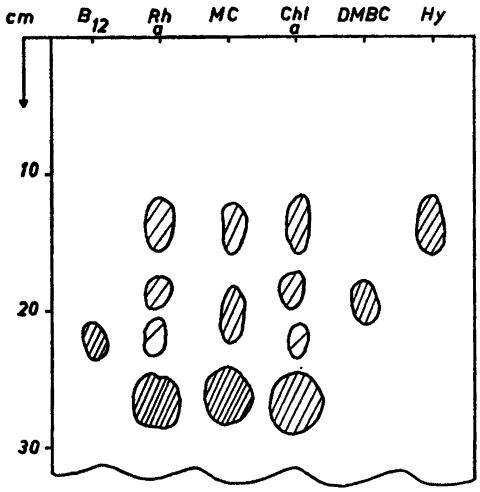

Fig. 6. Re-chromatography (*a*) of the fast-moving light-sensitive material separated from crude extracts from *Rhodosorus* (Rh) and *Chlorella* (Chl). Solvent III. MC, methylcobalamin.

placed on large agar plates seeded with *E. coli 113—3*. The position of the fast-moving light-sensitive band was localized in this way. This band was eluted from the corresponding segment of the paper sheet using distilled water and the eluate was freeze dried. Re-chromatography of the eluted band in solvent III alongside solutions of methyl-cobalamin treated in a similar way gave the pattern shown in Fig. 6. A large part of the activity was found in the position of the original fast-moving light-sensitive band, but a non-unconsiderable part of the activity was present in the positions corresponding to the other spots detected in the original extracts. Solutions of crystalline methylcobalamin treated in a way corresponding to that employed for the eluted bands gave rise to a similar chromatographic pattern (*cf.* Fig. 6).

DISCUSSION

The ever increasing number of corrinoids that have so far been described makes it difficult to identify them in crude microbial extracts by means of simple chromatographic and electrophoretic studies. The difficulty is further accentuated when the extracts are prepared under conditions favouring the stability of B_{12}-coenzymes. The presence of at least three forms of each corrinoid then can be expected; the coenzyme(s), and the aquo- and the cyano-derivatives. On the other hand, the assay of crude extracts secures a more complete detection of corrinoid compounds than can be expected after the available enrichment and purification procedures. The latter are always more or less selective with respect to different analogues (*cf.* Ref. 7). With certain materials, as is, *e.g.*, the case with algae, the bioautographic analysis of crude extracts is the only available technique, for the time being.

In order to facilitate the identification of B_{12}-substances extracted from algae certain available literature data (chromatographic mobilities of reference substances) and the results of the present investigation have been tabulated in Table 3. Most of the literature data quoted refers to the work of Pawelkiewicz *et al.*[13] and Zagalak and Pawelkiewicz [14] both of whom have employed a chromatographic system which has been found in this laboratory to be very useful for the separation of corrinoids from algae. It should be stressed that the literature data, which refer to compounds synthesized in either an enzymic [13] or a chemical [14] way, were obtained with relatively large amounts of the compounds, the so-called "visible spots", whereas the values concerning algal extracts were obtained by means of the bioautographic technique, *i.e.* using very small quantities (about 1 mμg) of the substances studied. Furthermore, the authors quoted carried out their chromatography at 24°C whereas our determinations were made at 37°C.

The chromatographic mobilities are given in relation to the "B_{12}-coenzyme", DMBC, *i.e.* the 5′-deoxyadenosyl derivative of vitamin B_{12}.

Inspection of Table 3 reveals certain regularities in the chromatographic mobilities of the corrinoid compounds. The light-sensitive derivatives of the 5′-deoxyadenosyl type move usually faster than the corresponding aquo-derivatives, but slower than the cyano compounds, whereas the methyl derivatives move considerably faster than the corresponding cyano derivatives.

Table 3. Chromatographic mobilities of certain corrinoids.
Our determinations[c] ($R_{DMBC} \pm 0.1$) and literature references (quoted with two decimals as in the original papers).[a]
Solvent: butanol:propan-2-ol:acetic acid:water (100:70:1:100, by vol.)[a] descending technique.

	Light sensitive derivative[d]	Aquo-derivative	Cyano-derivative
Vitamin B_{12}		0.7[c]	1.2[c]
Cobinamide (Factor B)	1.22[e]		1.71[b]
Aquocobalamin		0.7[c]	1.2[c]
Co-5′-dAdo-cobamide (DMBC)	1.00[a,c]	0.7[c]	1.2[c]
Co-5′-dAdo-cobinamide	1.08[a]		
Co-methyl-DMB-cobamide	1.62[a]		
	1.6[c]		
Co-methyl-cobinamide	1.62[a]		
ψ-B_{12}	0.47[b]	0.5[c]	0.8[c]
Factor A	0.54[b]	0.5[c]	1.0[c]
Factor III	0.75[b]	0.6[c]	1.0[c]
5-methyl-benzimid.-cobamide	0.86[b]	0.7[c]	1.1[c]
2-methyl-SH-aden.-cobamide		0.6[c]	0.8[c]
Factor Ib (cobinamide phosphateribose)			1.6[c]
Vitamin B_{12p}	1.22[b]		1.71[b]
y 1 (cobinamide guanosine diphosphate)	0.32[b]		

[a] Zagalak & Pawelkiewicz (1964), synthetic compounds; [b] recalculated from Pawelkiewicz *et al.* (1961), obtained by enzymic *in vitro* synthesis; [c] Whatman 3MM paper, 18 h at 37°C, bioautography on *E. coli* 113−3; [d] if not otherwise stated, the "light sensitive" derivative usually refers to the 5′-deoxyadenosyl derivative; [e] identical with vitamin B_{12p} of Pawelkiewicz.

Judging from the chromatographic mobilities the fast-moving light-sensitive spot ($R_{DMBC} = 1.5$), observed in extracts from *Chlorella* (only grown in the presence of B_{12}, *cf.* Table 1 and Fig. 1), in the extracts from *Rhodosorus* and occasionally also in extracts from *Anabaena* (*cf.* Table 1) would be practically indistinguishable from methyl-cobalamin in solvent III ($R_{DMBC} = 1.6$), whereas chromatography in solvent I could possibly result in a separation. In agreement with these data, chromatograms of algal extracts mixed with solutions of crystalline methyl-cobalamin gave no separation of the light-sensitive material and of the reference substance when solvent III was employed but there was some indication that these two materials are different on chromatograms run in solvent I (*cf.* Figs. 5:1 and 5:2). It seems therefore probable that the fast-moving light-sensitive material detected in extracts from all three algal species is different from methyl-cobalamin, but it may represent a methyl derivative of some B_{12}-analogue. The isolation of methyl-cobalamin from a natural source material has been achieved only recently [21] although it has been known for the past few years that the corresponding synthetic compound [22] can serve as a methyl donor in certain enzymic *in vitro* systems, such as the formation of methionine from homocysteine by liver extracts [23] and of methane by extracts of methane bacteria. [24,25] It is interesting

to consider in this connection the recent finding that the synthetic compound methyl-cobinamide (methylated Factor B) also functions as methyl donor in *in vitro* systems; in certain systems the methylating activity of methyl-cobinamide is much larger than that of methyl-cobalamin.[26] Certain other B_{12}-analogues were shown to stimulate *in vitro* the methyl-folate-H_4 and methyl-B_{12} transferase activities which result in the formation of methionine from homocysteine.[27] Both synthetic compounds are light-sensitive and also sensitive to cyanide treatment, as are the 5′-deoxyadenosyl cobamides.

The other light-sensitive spot observed mainly in extracts from *Anabaena* and a corresponding spot detected in the extracts from *Chlorella* (R_{DMBC} = 1.2 in solvent III, *cf.* Table 1, Figs. 1 and 4) is difficult to identify. Its chromatographic mobility is similar to that of a light-sensitive derivative of cobinamide or Factor B or vitamin B_{12p}, a derivative of the 5′-deoxyadenosyl-type [13,28,29] (*cf.* Table 3), *i.e.* a "nucleotide lacking coenzyme". The large proportion of such light-sensitive corrinoids in *Anabaena* (*cf.* Fig. 4) indicates that they may play some vital part in the enzymic activities of this organism. It should be reminded in this connection, however, that nucleotide lacking coenzymes isolated from bacteria are inactive in the glutamate mutase assay [11] (*cf.* p. 348).

It is interesting to note that DMBC was only observed in extracts from *Chlorella* and *Rhodosorus* when the algae were grown with B_{12} in the medium and was not found in the other cultures. However, with view to the multiple metabolic effects of vitamin B_{12}, not all of which seem to be dependent on the coenzymes of the DMBC type (5′-deoxyadenosyl-cobamides), the possibility must be considered that other coenzyme forms of corrinoids may also occur in nature.

Methionine has been included as a reference substance because it can replace B_{12} as growth factor for *E. coli 113—3*, albeit in concentrations 10^4 times higher than those of the vitamin. It is seen in Table 1 that the chromatographic mobility of methionine in solvent II and III is close to that of methyl-cobalamin, whereas in solvent I the mobility of methionine is considerably greater than the mobility of any of the corrinoid compounds. It can therefore be concluded that the *E. coli 113—3* activity of algal extracts was for the main part due to corrinoids and not to methionine.

Earlier work on corrinoids in algae, which, as mentioned in the introduction, revealed the presence of B_{12}-active compounds in this plant material, involved extraction in the presence of cyanide,[3] a procedure which results in the conversion of the corrinoid compounds to their respective cyano-derivatives. It appears from the present investigation that all three species of unicellular algae that have been investigated not only contain several corrinoids, but also have the ability to convert exogenously supplied cyano-forms of vitamin B_{12} or Factor III to other derivatives. The light-sensitive character of some of the latter compounds and a similar feature in certain of the corrinoids present in algae grown without an exogenous source of B_{12} indicate that the corrinoid derivatives detected in algal cell extracts may play an essential physiological, and perhaps coenzyme, function. The fact that such corrinoids were found in extracts from cells grown under rigorously sterile conditions lends further support to this view.

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