Mangiferin Trimethyl Ether—A suspension of mangiferin (30 mg.) in 2 ml. methanol and 8 ml. ether was treated with an excess of ethereal diazomethane following the procedure of Hawthorne and co-workers (7). After twice being precipitated from aqueous acetone, the product had m.p. 298–299° and provided infrared and ultraviolet spectra identical with those of an authentic sample. Literature m.p. 298–299° (7).

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Keyphrases

Hiptage madablota
Mangiferin—isolation, identification
Biological activity—mangiferin
IR spectrophotometry—structure
UV spectrophotometry—structure

Deficiency of Vitamin B_{12} in *Chlorella*

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Repeated systematic attempts to detect vitamin B_{12} in two species of *Chlorella* at different times during the culture growth period failed to reveal consistent or significant evidence of B_{12} or B_{12}-like activity. The results are discussed in terms of other reports in the literature.

As PART of a continuing investigation of the vitamin content of two *Chlorella* species (1–4), extracts of *C. vulgaris* and of *C. pyrenoidosa* have been assayed for vitamin B_{12} (cyanocobalamin and related factors). This seemed of particular interest in view of the conflicting reports in the literature concerning occurrence of the vitamin in chlorophyllous plants—especially the algae and particularly the Chlorococcales.

In an important review of vitamin B_{12} in the metabolism of microorganisms, Ford and Hutner (5) state "B_{12} . . . is not present in green plants and yeast; it is synthesized by bacteria and probably by blue-green, brown, and red algae, but not by green algae." Others have suggested that B_{12} found associated with seaweeds (whether red, brown, or green) can be attributed to bacteria living epiphytically on the algae (6, 7) or to bacterial contaminants in the distilled water used in preparing specimens for assay (8).

Referring specifically to *Chlorella* (species unspecified), Hutner (9) commented on the lack of B_{12} in the organism while, in contrast, Hashimoto reported significant levels of the vitamin (av. 61 μ mcg./mg. dry wt.) in "pure cultured" *Chlorella ellipsoidea* and he considered this a sufficiently high level to warrant further study (10).

METHODS

Chlorella vulgaris and *C. pyrenoidosa* were cultured aseptically by both the column and the round-bottom flask techniques described previously (11): light intensity in the former was 600 ftc. and in the latter 1250 ftc. Column cultures were harvested at different times from Day 5 to Day 21; flask cultures were harvested at intervals from 3 weeks to 10 weeks postinoculation.

Assays were performed using *Euglena gracilis* (ATCC 12716) and *Ochromonas malhamensis* (ATCC 11532) in the appropriate Difco media. Acid-cleaned glassware and fresh glass-redistilled

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water were used for all assays. Standard curves were determined in each assay using USP cyanocobalamin reference standard.

Three basic extraction procedures were employed: (*a*) water extraction with heat, (*b*) refluxing with 70% isopropanol, and (*c*) extraction with perchloric acid. A number of variations of these were tried including (*a*) addition of sodium cyanide (10 mg./ml.), (*b*) buffering with 0.01 *N* sodium acetate or adjusting pH with HCl, (*c*) heating at 90° or autoclaving, (*d*) subsequent digestion with papain, and (*e*) preliminary mincing in a mortar with crystalon abrasive.

No single procedure seemed consistently or distinctly superior for releasing B_{12} (if present) from the algae. In some instances extracts prepared with sodium acetate seemed to be slightly inhibitory for the *Euglena* test organism; use of isopropanol resulted in an extract containing lipids and other cell material that interfered with turbidity readings during actual assay; the perchloric acid technique resulted in extracts significantly inhibitory to growth of *Euglena*, due to inability to completely remove the perchloric acid, and the papain technique gave a high blank reading that overshadowed any possible B_{12} in *Chlorella*.

Therefore, the method recommended by the Analytical Methods Committee (12) was used routinely. In this procedure a suspension of cells with 0.01% NaCN (pH 4.5 with HCl) was heated in a water bath at 90° for 30 min. Recovery of added B_{12} was approximately 110%.

RESULTS

In seven different experiments between October 1966 and August 1967, each entailing several assays of extracts prepared from one to five harvests made at different times during the culture period, no significant or consistent levels of B_{12} or B_{12}-like activity were detected. Most frequently no B_{12} was found. Low levels (0.1 to 5 μmcg./mg. dry wt.) were found sporadically, generally in cultures 14 days or more postinoculation.

Although aseptic techniques are employed in setting up the algal cultures, the danger of bacterial contamination having occurred during subsequent manipulation cannot be totally eliminated, especially for older cultures. Therefore, the possibility of low-level bacterial contamination as the cultures age must be considered as a possible source of B_{12}.

Moreover, the B_{12} levels found were generally so low that often they were within the range of error inherent in the extraction and assay procedures.

The possibility exists that extraction procedures were not adequate for releasing B_{12} from the *Chlorella* cells but, in view of the variety of procedures and modifications employed, it seems unlikely that all of the methods would have been totally inadequate. Nonetheless, the possibility that B_{12} is protein bound and not readily released or that the tough *Chlorella* cell wall prevents release should not be overlooked.

Other possibilities are (*a*) the synthetic medium employed for culturing the algae precluded or interfered with biosynthesis of the vitamin, and/or (*b*) B_{12} is synthesized but so quickly transferred in one or more various synthetic pathways that it never accumulates in assayable levels.

However, under the conditions employed for culture and for the variety of techniques for extracting the cells and assaying the extracts, the authors have been unable to demonstrate the presence of B_{12} or B_{12}-like activity in *Chlorella vulgaris* and *C. pyrenoidosa*.

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Keyphrases

Vitamin B_{12}
Chlorella species—vitamin B_{12} content
Cultures—column, round-bottom flask techniques