Auxotrophy and Organic Compounds in the Nutrition of Marine Phytoplankton

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The artificial cultivation of marine planktonic diatoms dates from the studies of E. J. Allen at Plymouth, who maintained 'persistent' (i.e. 'unialgal', or 'bacterized') cultures of several species in enriched natural sea water (Allen & Nelson, 1910; Allen, 1914). Defined media failed to support the diatoms, but a very small addition of natural sea water or algal extract sufficed to make good the deficiency. Allen inferred the presence in sea water of an organic substance active in catalytic amounts, which he likened to the then recently discovered 'vitamine' from the husk of rice. One result of Allen's studies was the recognition that sea waters of different origin varied greatly in their ability to promote the growth of algae. Trace metals such as manganese and iron were involved but, as Harvey (1939) showed, they could by no means always account for all the observed effects, since certain compounds containing divalent sulphur were needed to complete the enrichment of infertile water. Experiments on settling of pelagic larvae were later to lead Wilson (1951) to similar thoughts concerning the biological activity of different waters and to conclude that the activity—in this case rendering a substratum suitable for settlement—was due chiefly to the presence of living bacteria (Wilson, 1954).

Allen's invocation of what is now called auxotrophy was out of harmony with contemporary views on algal nutrition, for the work of Molisch (1895, 1896), Benecke (1898) and Chodat (1913) had given rise to the belief that algae were by and large autotrophic like higher plants (though facultative chemotrophs were known). Furthermore, the earliest critical study with an axenic culture of a marine diatom (Peach & Drummond, 1924) was on the supra-littoral *Phaeodactylum tricornutum* (syn. *Nitzschia closterium* forma *minutissima*) which, contrary to the rule, as it has since proved, grows on the simplest mineral media. The subsequent wide use of this diatom in physiological studies has tended to obscure the import of Allen and Harvey's observations. Meanwhile, a new generation of algal physiologists was coming to the conclusion that algae were not as exclusively autotrophic as had been thought, and that the error had been due to a selection of autotrophs by the isolation methods previously in use (Pringsheim, 1946). The introduction of the use of soil extract by Pringsheim (1912, 1926) greatly increased the range of organisms which it was possible to grow in axenic culture, with the result that the extent to which auxotrophy is prevalent among the lower groups of algae gradually became evident (see Provasoli & Pintner, 1953). The culture medium familiar to marine biologists by the name of 'Erdschreiber' (Føyn, 1934) owes its success largely to the presence of soil extract.

A first step to an understanding of the government of a micro-organism in the field is an analysis of the properties of any natural substances which are required to support axenic growth *in vitro*. The benefit of soil extract, for example (or indeed natural sea water), might in any instance be derived from any or all of a number of properties (Pringsheim, 1935), among which may be listed: (*a*) control of trace metals; (*b*) pH buffering; (*c*) supply of organic micronutrients (satisfaction of auxotrophic requirements); (*d*) other quasi-physical properties such as poising or neutralizing of toxicity. Wilson's observations on the effect of bacteria on the settlement of Ophelia have been referred to; they parallel the experiences, too numerous to instance, of those who have attempted to cultivate fastidious algae. The difficulty of establishing axenic cultures of members of the phytoplankton is evidence of a dependence upon bacterial products (the problem of actual purification usually not being great). This, however, does not necessarily indicate auxotrophy on the part of the alga, since bacterial products may have any of the properties listed above for soil extract; properties which under given circumstances would cause them to influence algal growth.

Culture solutions with trace metals nicely balanced against the metal chelator ethylenediamine tetra-acetic acid (EDTA; Hutner *et al.* 1950; Provasoli & Pintner, 1953; Droop, 1955*a*) and containing tris-(hydroxy-methyl)-amino-methane (TRIS) as a pH buffer (Provasoli, McLaughlin & Pintner, 1954; Droop, 1955*b*) have effectively replaced soil extract so far as these functions are concerned, and have facilitated the cataloguing of vitamin requirements of a number of organisms previously dependent upon soil or other extracts. Thiamine and particularly cobalamin (vitamin B_{12}) are the B vitamins most frequently cited as being required by algal flagellates and diatoms. In many cases soil extract and natural sea water can be completely replaced by these two vitamins, appropriate cation mixtures and a pH buffer. Biotin has been recorded among the requirements of the freshwater *Ochromonas malhamensis* (Hutner, Provasoli & Filfus, 1953). It appears that heterotrophy other than auxotrophy is rare among the few marine pigmented species studied nutritionally. *Hemiselmis virescens* is an example of an alga dependent upon amino nitrogen (preferably glycine), while some others are facultative in this respect (Droop, 1955*a*). All, including those which are phagotrophic, appear to be obligate phototrophs (at pH 8 at any rate). The colourless species of course are necessarily chemotrophic and the non-phagotrophs among them (e.g. *Peridinium depressum*) require dissolved organic compounds capable of providing energy. None of the latter group has yet been cultivated free of bacteria.

The identification of thiamine as one of the active principles in soil extract was made by Lwoff & Lederer (1935); since then a need for thiamine has been recorded in a number of Chlorophyta, Euglenophyta, Pyrrhophyta and Chrysophyta (cf. Provasoli & Pintner, 1953). Marine species include several chrysomonads (Droop, 1954), but the effect of thiamine for them was merely stimulatory. In point of fact, an absolute requirement for thiamine in the part of any marine phytoplankton has yet to be established.

The situation with vitamin B_{12} is less ambiguous since the requirement, when present, is absolute and demonstrable by a clear dose/response relation. The isolation of the crystalline vitamin from ox liver (Smith, 1948) was quickly followed by its identification by Hutner and his co-workers with factors needed by the flagellates *Euglena gracilis* and *Ochromonas malhamensis* (Hutner *et al.* 1949, 1953). This led to recognition of a need for vitamin B_{12} on the part of an impressive number of marine and freshwater algae and flagellates which had previously been cultivated only with the aid of soil, liver or other natural extracts (Provasoli & Pintner, 1953; R. A. Lewin, 1954; Droop, 1954, 1955a, b; Sweeney, 1954). Soil extract may contain considerable amounts of vitamin B_{12}: for example, 1–8 μg./l. cold water extract (Robbins, Hervey & Stebbins, 1950) or 9–60 μg./l. of hot alkaline extract (Droop, unpublished). Vitamin B_{12} requirers represent a large proportion of the marine organisms which have so far been grown in axenic culture. Tables 1–3 are not by any means representative of the phytoplankton, either oceanic or neritic, but they do contain some pelagic species, and an interest by marine ecologists in the distribution of vitamin B_{12} seems to be well justified.

Table 1. *Marine algae known to require vitamin B_{12}*

Dinophyceae
    Amphidinium klebsii   Provasoli, unpublished
    A. rhynococephalum   Provasoli, unpublished
    *Exuviaella cassubica   Provasoli, unpublished
    *Gymnodinium splendens   Sweeney, 1954
    Gyrodinium californicum   Provasoli, unpublished
    Peridinium balticum   Provasoli, unpublished
    P. chattoni   Provasoli, unpublished
    *Prorocentrum micans   Droop, unpublished

Cryptophyceae
    Hemiselmis virescens   Droop, 1955a

Chrysophyceae
    *Isochrysis galbana   Provasoli, unpublished
    Microglena arenicola   Droop, 1955a
    Monochrysis lutheri   Droop, 1954, 1955c
    Prymnesium parvum   Droop, 1954
    Syracosphaera carterae   Provasoli & Pintner, 1953
    S. elongata   Droop, 1954

Bacillariophyceae
    Amphora perpusilla   Hutner & Provasoli, 1953
    *Skeletonema costatum   Droop, 1955b

Chlorophyceae
    Brachiomonas submarina   Hutner, unpublished
    Stichococcus sp.   Lewin, 1954

Cyanophyceae
    Phormidium persicinum   Provasoli & Pintner, 1954

* Pelagic species.

The numerous analogues of vitamin B_{12}, of varying activity for different organisms (Kon, 1955; Ford & Hutner, 1955), also deserve the attention of ecologists, in so far as these analogues may be shown to occur naturally and to evince a response in any organism. The pattern of specificity of several species

represented in Table 1 was studied by L. Provasoli (Haskins Laboratories, New York) and myself with the aid of vitamin B_{12}-like compounds isolated by E. S. Holdsworth and J. E. Ford of the National Institute for Research in Dairying, Shinfield, near Reading, Berkshire (Table 4). It appears that one or other of the specificity patterns known in other organisms is shown by most of these marine algae. Of the fourteen species tested, eight responded only to the

Table 2. *Some marine algae known not to require vitamin B_{12}*

Cryptophyceae
　　Rhodomonas lens　　　　　　　　Provasoli, unpublished

Chrysophyceae
　　Stichochrysis sp.　　　　　　　　Hutner, unpublished

Bacillariophyceae
　　Phaeodactylum tricornutum　　　Peach & Drummond, 1924

Chlorophyceae
　　Dunaliella spp.　　　　　　　　　Hutner, unpublished
　　Nannochloris oculata　　　　　　Droop, 1955a
　　Prasiola stipitata　　　　　　　　Lewin, 1955

Phaeophyceae
　　Ectocarpus parasitica　　　　　　Hutner, unpublished
　　Waerniella lucifuga　　　　　　　Droop, unpublished

Rhodophyceae
　　Porphyridium cruentum

* Pelagic species.

Table 3. *Some marine algae in axenic culture for which data are wanting*

Dinophyceae
　　Glenodinium foliacium　　　　　　Droop, unpublished
　　Peridinium trochoideum　　　　　Droop, unpublished
　　Oxyrrhis marina　　　　　　　　　Droop, unpublished

Chrysophyceae
　　Mallomonas epithallatia　　　　　Droop, unpublished

Baccillariophyceae
　　Coscinoscira polychorda　　　　　Droop, unpublished
　　Thalassiosira gravida　　　　　　Provasoli, unpublished
　　Stephanopyxis turris　　　　　　　Provasoli, unpublished

* Pelagic species.

vitamin proper, B_{12} III, and two artificial analogues (mammalian or Ochromonas pattern); one responded to all factors with the exception of the nucleotide-free factor B (*Lactobacillus leichmannii* or Euglena pattern); and three responded to all, including factor B (*Escherichia coli* pattern). It is possibly significant that the two diatoms are in this group. Two of the dinoflagellates were exceptional in being able to differentiate between pseudo-B_{12} (containing adenine) and factor A (containing 2-methyladenine), responding to the latter and not the former. It is early to generalize; nevertheless, judging by these first results it is likely that the analogues of vitamin B_{12} will have an importance equal to that of the vitamin.

Since most of the marine algae whose nutritional requirements are known are estuarine, littoral or supralittoral and not pelagic, it is impossible yet to assess the role of vitamin B_{12} in the ecology of phytoplankton in general. A requirement for vitamin B_{12} confined to neritic species and distinguishing them from their oceanic relatives might be a major factor in regional distribution. On the other hand, a seasonal control might be exerted on oceanic forms which require vitamin B_{12} in any region where the concentration decreases

Table 4. *Specificity towards vitamin B_{12}-like factors*

Organism	B₁₂ (5, 6-dimethyl-benziminazole)	5, 6-dichlorobenzi-minazole analogue	Benziminazole analogue	B₁₂ III (Factor I)	Factor A (2-methyladenine)	Factor H (2-methylhypo-xanthine)	Pseudo-B₁₂ (adenine)	Factor B (No nucleotide)
Prymnesium parvum	+	+	+	+	0	0	0	0
Microglena arenicola	+	+	+	+	0	0	0	0
Syracosphaera elongata	+	+	+	+	0	0	0	0
Isochrysis galbana	+	+	+	+	0	0	0	0
Hemiselmis virescens	+	+	+	+	0	0	0	0
Gyrodinium californicum	+	+	+	+	0	0	0	0
Gyrodinium sp.	+	+	+	+	0	0	0	0
Amphidinium klebsii	+	+	+	+	+	+	0	0
A. rhyncocephalum	+	+	+	+	+	+	0	0
Monochrysis lutheri	+	+	+	+	+	+	+	0
Amphora perpusilla	+	+	+	+	+	+	+	+
Skeletonema costatum	+		+	+			+	+
Phormidium persicinum	+	+	+	+	+	+	+	+

+ = activity 25 % or more; 0 = activity less than 1 %; as compared with vitamin B_{12}.

sufficiently, a contingency more probable in the oceans than in coastal waters. Little has been done up to now on the seasonal and spatial distribution of vitamin B_{12}. The first assays of the content of coastal waters (Lewin, 1954; Droop, 1954, 1955 c) suggested figures of 5–10 mμg./l., which were high enough to support the heaviest known plankton crops; it appeared likely that vitamin B_{12} would not be an important ecological factor after all. But more recently samples of water from the north North Sea and Norwegian Deeps taken over a period of 10 months have been assayed by Cowey (1956) who recorded values as little as 0·1 mμg./l.; these figures are low enough to hold promise of significant spatial and temporal differentials.

Marine vitamin B_{12} is likely to be mainly of bacterial origin (Hall *et al.* 1950; Lochhead & Thexton, 1951; Ericson & Lewis, 1953), though reports of its production by Chlorella (Robbins, Hervey & Stebbins, 1951; Hashimoto, 1954; Brown, Cuthbertson & Fogg, 1956) indicate that the non-auxotrophic members of the phytoplankton and even benthic algae should not be entirely dismissed. Since marine bacteria have been found mainly associated with par-

ticulate matter (ZoBell, 1946) it is to be expected that coastal and estuarine areas and regions of upwelling will have the heaviest turnover.

To turn now to the sulphur requirements of diatoms; Harvey (1939) found that any of a number of compounds containing organic sulphur (cystine, glutathione, biotin, thiamine) were required for vigorous growth of *Ditylum brightwellii* in artificial sea water. Matudaira (1942) added inorganic sulphide to this list for *Skeletonema costatum*, and Harvey (1955) thiourea. All these experiments are to some extent open to criticism as they were carried out with bacterized cultures. Nevertheless, they were confirmed in principle with axenic cultures of the freshwater *Navicula pelliculosa* by J. C. Lewin (1954), who found that the washed diatoms were unable to assimilate silicon unless a compound containing divalent sulphur was added to the medium. Furthermore, sulphate was unable to restore their ability to assimilate but sulphate plus a reducing agent such as ascorbic acid did so.

A bacteria-free strain of *Skeletonema costatum* has now been maintained at Millport for nearly two years (Droop, 1955b). The defined medium contained thiamine, but on two occasions the diatom was passed through five transfers in the medium with thiamine omitted, without statistically-significant diminution in growth. It seems that there is no absolute requirement for thiamine or other organic sulphur compound. J. C. Lewin's (1954) experiment with ascorbic acid and sulphate suggests that the question of divalent sulphur might be tied up with redox phenomena. Some recent experiments at Millport support this view, as they show that the need for divalent sulphur is largely overcome when certain conditions of aeration are observed. For instance, vigorously aerated cultures of *S. costatum* require 3 mg. $Na_2S.9H_2O$/l., while still (non-aerated) cultures will not tolerate more than 1·0 mg./l. unless the medium has been allowed to stand some days before being used, and they grow as well without any added sulphide if the medium has not been allowed to stand too long. One finds the effect of sulphide is greatest when the inoculum has been taken from stationary phase cultures, and least when from logarithmic phase cultures. Harvey (1939) remarked the importance of the state of the inoculum. It seems that the sensitive period is the lag phase. The interdependence of sulphur requirements and oxygen tension may be reflecting the lability of the thiol radical in redox systems (the radical itself constituting the requirement) or may, on the other hand, mean that the function of these thiols is merely to maintain a critical redox potential at the cell surface.

In conclusion, I quote Provasoli & Pintner (1953); their statement 'it is a reasonable assumption that if an organism requires a growth factor *in vitro* then this metabolite or its physiological equivalent should be found in the environment' is an article of faith which has led to some useful results with vitamin B_{12} and will continue to do so with other compounds involving strictly trophic requirements. But compounds whose activity is physical rather than trophic (such as, possibly, the thiols) present a rather different problem, since their 'physiological equivalents' are likely to be numerous and their effects ill-defined and complex, and in some cases, no doubt, only of consequence in artificial cultures. Nevertheless, I believe these compounds

will prove as relevant to what Lucas (1949) has termed 'non-predatory' relationships among phytoplankton as any which are more simply trophically active.

REFERENCES

ALLEN, E. J. (1914). On the culture of the plankton diatom *Thalassiosira gravida* Cleve, in artificial sea water. *J. mar. biol. Ass. U.K.* **10**, 417.

ALLEN, E. J. & NELSON, E. W. (1910). On artificial culture of marine plankton organisms. *J. mar. biol. Ass. U.K.* **8**, 421.

BENECKE, W. (1898). Über Kulturbedingungen einiger Algen. *Bot. Ztg,* **56**, 83.

BROWN, F., CUTHBERTSON, W. F. J. & FOGG, G. E. (1956). Vitamin B_{12} activity of *Chlorella vulgaris* Beij. and *Anabaena cylindrica* Lemm. *Nature, Lond.* **177**, 188.

CHODAT, R. (1913). *Monographe d'algues en culture pure.* Berne: K. J. Wyss.

COWEY, C. B. (1956). *J. mar. biol. Ass., U.K.* **35**, 609.

DROOP, M. R. (1954). Cobalamin requirement in Chrysophyceae. *Nature, Lond.* **174**, 520.

DROOP, M. R. (1955a). Some new supra-littoral Protista. *J. mar. biol. Ass. U.K.* **34**, 233.

DROOP, M. R. (1955b). A pelagic marine diatom requiring cobalamin. *J. mar. biol. Ass. U.K.* **34**, 229.

DROOP, M. R. (1955c). A suggested method for the assay of vitamin B_{12} in sea water. *J. mar. biol. Ass. U.K.* **34**, 435.

ERICSON, L. E. & LEWIS, L. (1953). On the occurrence of vitamin B_{12}-factors in marine algae. *Ark. Kemi.* **6**, 427.

FORD, J. E. & HUTNER, S. H. (1955). Role of vitamin B_{12} in the metabolism of microorganisms. *Vitam. & Horm.* **13**, 101.

FØYN, B. (1934). Lebenszyklus, Cytologie und Sexualität der Chlorophyceae *Cladophora suhriana* Kützing. *Arch. Protistenk.* **83**, 1.

HALL, H. H., BENJAMIN, J. C., BRICKER, H. M., GILL, R. J., HAYNES, W. C. & TSUCHIYA, H. M. (1950). A survey for B_{12} producing microorganisms. *Proc. Soc. Amer. Bact.* **50**, 21.

HARVEY, H. W. (1939). Substances controlling the growth of a diatom. *J. mar. biol. Ass. U.K.* **23**, 499.

HARVEY, H. W. (1955). *The Chemistry and Fertility of Sea Water.* Cambridge University Press.

HASHIMOTO, Y. (1954). Vitamin B_{12} in marine and freshwater algae. *J. Vitaminol.* **1**, 49.

HUTNER, S. H. & PROVASOLI, L. (1953). A pigmented marine diatom requiring vitamin B_{12} and uracil. *News Bull. Phycolog. Soc. Amer.* **6**, (no. 18), 7.

HUTNER, S. H., PROVASOLI, L. & FILFUS, J. (1953). Nutrition of some phagotrophic freshwater Chrysomonads. *Ann. N.Y. Acad. Sci.* **56**, 852.

HUTNER, S. H., PROVASOLI, L., SCHATZ, A. & HASKINS, C. P. (1950). Some approaches to the study of the role of metals in the metabolism of micro-organisms. *Proc. Amer. phil. Soc.* **94**, 152.

HUTNER, S. H., PROVASOLI, L., STOCKSTAD, E. L. R., HOFFMANN, C. E., BELT, M., FRANKLIN, A. L. & JUKES, T. H. (1949). Assay of antipernicious anemia factor with *Euglena*. *Proc. Soc. exp. Biol., N.Y.* **70**, 118.

KON, S. K. (1955). Other factors related to vitamin B_{12}. *Biochem. Soc. Symp.* **13**, 17.

LEWIN, J. C. (1954). Silicon metabolism in diatoms. 1. Evidence for the role of reduced sulfur compounds in silicon utilization. *J. gen. Physiol.* **37**, 589.

LEWIN, R. A. (1954). A marine *Stichococcus* sp. which requires Vitamin B_{12} (cobalamin). *J. gen. Microbiol.* **10**, 93.

LEWIN, R. A. (1955). Culture of *Prasiola stipitata* Suhr. *Canad. J. Res.* **33**, 5.

LOCHHEAD, A. G. & THEXTON, R. H. (1951). Vitamin B_{12} as a growth factor for soil bacteria. *Nature, Lond.* **167**, 1034.

LUCAS, C. E. (1949). External metabolites and ecological adaptations. *Symp. Soc. exp. Biol.* **3**, 336.

LWOFF, A. & LEDERER, E. (1935). Remarques sur 'l'extrait de terre' envisagé comme facteur de croissance pour les flagellés. *C.R. Soc. Biol., Paris,* **119**, 971.

MATUDAIRA, T. (1942). On inorganic sulphides as a growth promoting ingredient for diatom. *Proc. Imp. Acad. Japan,* **18**, 107.

MOLISCH, H. (1895). Die Ernährung der Algen. I. Süsswasseralgen. *S.B. öst. Akad. Wiss.* (Abt. I), **104**, 783.

MOLISCH, H. (1896). Die Ernährung der Algen. II. Süsswasseralgen. *S.B. öst. Akad. Wiss.* (Abt. I), **105**, 633.

PEACH, E. A. & DRUMMOND, J. C. (1924). On the culture of the marine diatom *Nitzschia closterium* (F.) *minutissima* in artificial sea water. *Biochem. J.* **18**, 464.

PRINGSHEIM, E. G. (1912). Die Kultur von Algen in Agar. *Beitr. Biol. Pfl.* **11**, 305.

PRINGSHEIM, E. G. (1926). Methoden und Erfahrungen. *Beitr. Biol. Pfl.* **14**, 283.

PRINGSHEIM, E. G. (1935). Wuchstoffe im Erdboden. *Naturwissenschaften,* **12**, 197.

PRINGSHEIM, E. G. (1946). *Pure Cultures of Algae.* Cambridge University Press.

PROVASOLI, L., McLAUGHLIN, J. J. A. & PINTNER, I. J. (1954). Relative and limiting concentrations of major mineral constituents for the growth of algal flagellates. *Trans. N.Y. Acad. Sci.* **16**, 412.

PROVASOLI, L. & PINTNER, I. J. (1953). Ecological implications of *in vitro* nutritional requirements of algal flagellates. *Ann. N.Y. Acad. Sci.* **56**, 839.

PROVASOLI, L. & PINTNER, I. J. (1954). Cultural characteristics of *Phormidium persicinum,* an auxotrophic marine red pigmented blue green alga. *Rapp. Comm. Congr. Int. Bot. Paris,* **8** (Sect. 17), 39.

ROBBINS, W. J., HERVEY, A. & STEBBINS, M. E. (1950). Studies on *Euglena* and Vitamin B_{12}. *Bull. Torrey bot. Cl.* **77**, 423.

ROBBINS, W. J., HERVEY, A. & STEBBINS, M. E. (1951). Further observations on *Euglena* and B_{12}. *Bull. Torrey bot. Cl.* **78**, 363.

SMITH, E. L. (1948). Purification of anti-pernicious anemia factors from liver. *Nature, Lond.* **161**, 638.

SWEENEY, B. M. (1954). *Gymnodinium splendens,* a marine dinoflagellate requiring vitamin B_{12}. *Amer. J. Bot.* **41**, 821.

WILSON, D. P. (1951). A biological difference between natural sea waters. *J. mar. biol. Ass. U.K.* **30**, 1.

WILSON, D. P. (1954). The attractive factor in the settlement of *Ophelia bicornis* Savigny. *J. mar. biol. Ass. U.K.* **33**, 361.

ZOBELL, C. E. (1946). *Marine Microbiology.* Waltham, Mass.: Chronica Botanica.