THE BORON NUTRITION OF THE DIATOM, *CYLINDROTHECA FUSIFORMIS*, GROWN ON AGAR, AND THE BIOLOGICAL ACTIVITY OF SOME SUBSTITUTED PHENYLBORONIC ACIDS

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Summary

The boron requirement of the diatom *Cylindrotheca fusiformis* Reimann & Lewin, grown in liquid culture, was confirmed. Methods are described in which this requirement is also easily demonstrated on agar, when it is satisfied by 10^{-5}M boric acid. Using this agar technique, the ability of various organic (and other) boron compounds to elicit a growth response on boron-deficient agar media was studied. Aqueous solutions of germanium dioxide were inactive. The results are discussed in relation to the various hypotheses regarding the relationship between the chemical structure and biological activity of boron compounds. The results obtained are consistent with the "complexing hypothesis" for the biological activity of borate.

I. Introduction

There are many advantages, for investigations into details of nutritional metabolism, in using a unicellular organism. Thus the use of intact higher plants as material on which to investigate the possible biochemical basis for the boron requirement of higher plants has been abandoned by some investigators in favour of other less complex plant systems which have a boron requirement: Neales (1959, 1964*a*) has used excised flax and tomato roots continuously grown in sterile culture, and Yih, Hille, and Clark (1966) have used callus tissues derived from the pollen of *Ginkgo biloba* L. by Tulecke (1953). Lewin (1965) demonstrated that the marine diatom, *Cylindrotheca fusiformis* Reimann & Lewin, will multiply when grown in an artificial liquid saline medium only when borate is added. This latter work showed that this unicellular organism could be used in further attempts to develop a convenient biological system with a clearly defined boron requirement. One of the advantages of developing an agar-plating technique is that, if successful, it would conveniently allow the feeding of metabolites (and the visual assessment of their growth effect) possibly not synthesized in boron-deficient cultures. This method is analogous to the detection, by nutritional methods, of the biochemical nature of "deficient" mutants of *Neurospora* (Beadle and Tatum 1945), and is extensively used with bacterial mutants. The biochemical genetics of algae have been reviewed by Ebersold (1962).

This investigation had two objectives. First, using Lewin's (1965) demonstration of the boron requirement of *C. fusiformis*, to attempt to produce boron-deficient cultures of this diatom growing on solid agar media: and second, to use this system to investigate the ability of various boron compounds to provide the growth stimulus normally provided by boric acid.

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The more explicit aim of the latter part of this investigation was to attempt to ascertain which property of the boric acid molecule was necessary for it to have the ability to provide "boron" to a boron-deficient culture of *C. fusiformis*. The present hypothesis in this matter (hereafter referred to as the "complexing hypothesis") was first proposed by Hoagland (1944): namely that the biological activity of boric acid in plants was related to the *in vitro* ability of this molecule to complex with organic *cis*-polyols (reviewed by Zittle 1951; Skok 1958; Weigel 1963). This hypothesis has never been directly tested, but frequently tacitly assumed (Gauch and Dugger 1954). Support for this hypothesis is given by Skok (1957) who showed that aqueous solutions of Specpure germanium dioxide [with which organic polyols also complex (Weigel 1963)] would slightly and temporarily replace boric acid as a boron source for sunflower seedlings.

The "complexing hypothesis" can be directly tested using derivatives of boric acid in which various of the three or four –OH groups (boric acid forms the $[B(OH)_4]^-$ ion in aqueous media) are substituted by various groups, other than –OH. The ability of these compounds to promote the growth of an organism, otherwise deficient in boron, is a measure of their biological activity. This activity can then be compared with that of boric acid, which is the form of this element usually supplied to plants.

The experiments of Torssell (1956), who worked with several ring-substituted derivatives of phenylboronic acid, do not provide evidence of the ability of these compounds to provide boron to the plant, because the wheat root assay used did not show a growth response to boric acid—the control treatments without boron were not boron-deficient. Experiments of the required type were published, with a different aim, by Stanley and Lichtenberg (1963) using a pollen germination and pollen tube extension assay. However, these experiments are inconclusive when the biological activity of various boron compounds used are compared.

Thus the second part of this paper describes an investigation of the ability of various organic boron compounds to support the growth of *C. fusiformis* on boron-deficient agar plates. The compounds were specifically chosen to test the "complexing hypothesis" described above.

II. Methods

(*a*) *Plant Material*

A culture of *C. fusiformis* (Reimann and Lewin 1964) was most kindly supplied by Dr J. C. Lewin of the Scripps Institute of Oceanography, California. The designation of the culture received was "strain 13 of Watson". This organism has been maintained in this laboratory on agar slopes and propagated in liquid culture in a modification of Lewin's (1965) medium, described below. The structure of *C. fusiformis* has recently been described by Reimann and Lewin (1964) and Reimann, Lewin, and Volcani (1965).

(*b*) *Methods of Culture*

The standard boron-deficient liquid medium used was similar to that of Lewin (1965). The following modifications were introduced: (1) iron was added

as Fe^{3+}-EDTA (0·5 p.p.m. Fe); (2) silicon was added from a standardized solution of sodium silicate to give 15 p.p.m. silicon in the basal medium, and (3) the micronutrients used, with the exception of boron, were those of Hoagland and Arnon (1950) at one-third of the recommended concentration. Boron was added separately as a boric acid, and the water used was distilled from steam in a silica condenser.

In the first experiments, the aim of which was to confirm Lewin's (1965) results and to find the least concentration of boric acid which would support the optimal growth rate, the diatom was grown in 50 ml of liquid medium in 125 ml polypropylene flasks which were shaken in a water-bath held at 25°C, and illuminated from below. Boron was added as boric acid at various concentrations, and growth assessed by measuring cell number per unit volume of solution, using a haemocytometer.

In experiments in which the diatom was grown on agar, the standard medium was autoclaved in polypropylene flasks with 1% Oxoid Ionagar No. 2, batch 57 (Oxo Ltd., London), after which it was poured in 25-ml aliquots into sterile, 8·7-cm diameter, polystyrene petri dishes. Oxoid agar No. 3, and certain batches of Ionagar No. 2, were found to be unsuitable for this purpose, presumably because they contained appreciable quantities of boron (Plate 1, Fig. 6). When the medium had solidified, the plates were sprayed with a suspension of the diatom which had previously been washed and centrifuged three times in the boron-deficient medium. The diatom was then allowed to grow in light provided by Warm White or Growlux fluorescent tubes (*c.* 3000 lux) at a constant temperature of 25 ± 1°C. Two different methods have been used to add boron to the solid agar medium. In the first, the media were made up and autoclaved containing concentrations of boric acid ranging from 10^{-8} to 1M; in the second, microgram quantities of boric acid (or other boron compounds) were applied in 10-μl drops to the centre of boron-deficient plates which had previously been sprayed with a suspension of the diatom. In experiments of the latter type, it was sometimes found convenient to apply the 10-μl drops onto disks of washed and sterile Whatman No. 541 filter paper, 7 mm in diameter, also placed in the centre of the plate. These small quantities of boron compounds induced the growth of small colonies of the diatom in the centre of the plate.

III. Results

(*a*) *Boron Requirement of* C. fusiformis *Grown in Sterile Liquid Media*

The following six concentrations of boron (as boric acid) were used: 0, 0·001, 0·01, 0·1, 1·0, and 10·0 p.p.m. boron, and there were three replicates of each. In addition, one culture was similarly set up with no addition of boron, but in a much-used clean Pyrex glass flask. The flasks were then autoclaved and subsequently inoculated with 0·1 ml of a washed suspension of the diatom, giving an initial cell number count of $1{\cdot}4 \pm 0{\cdot}8 \times 10^4$ cell/ml. The flasks were then incubated in light, as already described. After 12 days the flasks with concentrations of more than 0·01 p.p.m. boron all contained dense cultures of the diatom, as also did the culture in the Pyrex flask to which no borate had been added (Plate 1, Fig. 1). Previous experiments had shown that the stationary phase of growth was reached after

8 days, as in Lewin's (1965) experiments. Cell counts on the twelfth day gave the results in Table 1.

It is evident that, under these experimental conditions, the boron requirement of *C. fusiformis* is satisfied by a concentration of boron intermediate between 0·01 and 0·1 p.p.m. Lewin's (1965) finding, that this diatom has a boron requirement, is thus confirmed. Autoclaving a boron-deficient solution in Pyrex glass flasks provides the boron necessary for optimal growth.

TABLE 1

EFFECTS OF CONCENTRATION OF BORON SUPPLIED AS BORIC ACID ON THE CELL NUMBER OF *CYLINDROTHECA FUSIFORMIS* GROWN IN LIQUID CULTURE FOR 12 DAYS

Each value is the mean of two independent haemocytometer counts on different drops of the same culture

Boron Concn. (p.p.m.)	No. of Cells per 0·1 mm³			Mean No. of Cells
	Replicate 1	Replicate 2	Replicate 3	
0	8·5	5·5	12·5	8·8
0·001	19·0	8·5	20·0	12·5
0·01	100·5	22·5	12·5	45·2
0·10	248·0	266·0	280·0	264·7
1·0	239·0	282·0	274·5	265·2
10·0	220·0	236·5	270·5	242·3

(*b*) *Boron Requirement of* C. fusiformis *and* Chlorella vulgaris *Grown on Solid Agar Media*

Agar media in petri dishes containing boric acid in concentrations ranging from 10^{-8} to 1M were made up and inoculated with the diatom, as already described. Two replicates of each treatment were used. They were then placed in light for 12 days, when the plates of one replicate were photographed (Plate 1, Fig. 2). The second replicate appeared identical to the first, and plates containing sufficient boric acid placed in the dark did not support any more growth than was apparent on the plates in which the agar contained boric acid at a concentration of 10^{-7}M or less (Plate 1, Fig. 2).

It was evident: (1) that the diatom would grow well on agar media, if illuminated, (2) that the boron requirement was most clearly demonstrated, (3) that this requirement was satisfied, from visual inspection, by concentrations of boric acid of 10^{-5}M (0·11 p.p.m. boron) or above, and (4) that concentrations of 0·1 and 1M boric acid (1082 and 10820 p.p.m. boron) were toxic to this diatom.

Using similar agar methods, and an inorganic medium based on that of Moore and Duggar (1949), it was not found possible to demonstrate a boron requirement for *Chlorella vulgaris* (Cambridge strain, N211-11H). Profuse growth of *C. vulgaris* took place in the presence or absence of boric acid. This is similar to the results of Bowen *et al.* (1965), who used four other strains of *C. vulgaris*

grown in liquid culture and were unable to detect a boron requirement for the growth of this green alga.

(c) *Effects of Adding Small Drops of Solutions of Boric Acid onto the Surface of Inoculated Boron-deficient Plates*

It was now possible to proceed to those experiments in which boric acid was added in small drops on the centre of boron-deficient inoculated plates. Growth took place over 15 days in each of the three experiments. Plate 1, Figure 3, shows the dimensions of the diatom colony which resulted from the application of 10 μl of a boric acid solution ($\equiv$ 100 p.p.m. boron) onto boron-deficient media made up in 1, 2, 3, and 5% agar, respectively. Plate 1, Figure 4, shows a similar experiment in which 10 μl of a boric acid solution ($\equiv$ 1000 p.p.m. boron) was used. It is evident that both agar concentration and the mass of boron added affect the size of the resultant colony: presumably due to the different diffusion rates and concentration gradients of borate established in the media containing different concentrations of agar.

Plate 1, Figure 5, shows the results of an experiment in which the same mass of borate was added (0·1 μmole), but contained in drops of different volumes, to four plates of the basal medium in 1% agar. It appears that the colony size is a function of the mass of borate added, and not the volume of the drop in which it was contained, which is insignificant compared to the volume of water held in the solid lattice of the agar gel.

(d) *Ability of Various Boron Compounds to Induce the Growth of* C. fusiformis *when Applied Locally to the Surface of Boron-deficient Media*

Having established that a localized colony of *C. fusiformis* formed in response to a localized application of boric acid, it was now possible to compare the activity of other boron compounds with equimolar quantities of boric acid. As well as sodium tetrafluoroborate and sodium tetraphenylborate, phenylboronic acid and the ring-substituted derivatives 2-, 3-, and 4-methoxy, 2,6-dimethoxy, 3- and 4-chloro, 4-bromo, 4-phenyl, and 4-phenoxyboronic acids were tested. Germanium dioxide, which forms complex hydrates and hydroxides in aqueous solution which complex further with diols (Weigel 1963), was also tested.

Where the compounds containing boron were water-soluble, 0·1 μmole of each was applied in solution to each of several filter paper disks. These were then air dried, and one was placed in the centre of both an inoculated boron-deficient plate and of an inoculated plate containing boron at 0·2 p.p.m. Compounds insoluble in water were dissolved in 80% acetone, and the same procedure followed. The advantage of this technique is that diffusion outward from the point of application induces a concentration gradient which may pass from being toxic to the growth of the diatom, through a range which will support growth (if the compound is active), to concentrations which are too small to induce the visible growth of the diatom on the boron-deficient agar medium. Furthermore, the inhibition of growth that is observed in the series of plates containing boron gives a clear indication of the extent of the concentration range which is phytotoxic, beyond which (on the

boron-deficient plates) growth should therefore be observed if the compound applied in the centre of the plate is active as a source of boron. Also the presence of a central "phytotoxic halo" on the plate with boron is unequivocal evidence that the compound in question is present in the agar of the boron-deficient plate. If no growth has been induced on the latter, whilst phytotoxicity is apparent on the former, then there is no doubt that the particular compound used is inactive as a source of boron.

The results of a typical experiment are shown in Plate 1, Figure 7. The compounds germanium dioxide (aqueous solution) and 4-chlorophenylboronic acid are phytotoxic to the growth of the diatom on the boron-containing plates. On the boron-deficient plates, boric acid and 4-methoxyphenylboronic acid are strongly active, 4-chlorophenylboronic acid is slightly so, and germanium dioxide and sodium tetrafluoroborate are inactive. This indicates: (1) that the presence of the radical $-B(OH)_2$ is necessary for a compound to support growth on an otherwise boron-deficient plate, (2) that chloro-substitution in the *meta* position on the benzene ring of phenylboronic acid makes it more toxic to growth than the similar methoxy

TABLE 2

EFFECT OF GERMANIUM DIOXIDE ON FLAX SEEDLINGS GROWN FROM THE EXPANDED COTYLEDON STAGE IN NUTRIENT CULTURE FOR 9 DAYS

Flax seedlings grown at 25°C under continuous light of *c.* 1000 f.c.

Concentration (M) of Germanium Dioxide in Nutrient Solution	Fresh Weight (mg) of Five Seedlings	
	Without Borate	With Borate (10^{-5}M boric acid)
Nil	591	2000
5×10^{-8}	588	1953
5×10^{-7}	618	2066
5×10^{-6}	570	1734
5×10^{-5}	269	554
Mean	527 ± 65	1661 ± 282

compound, and (3) that germanium dioxide in aqueous solution is in toxic concentration over a small area of the boron-containing plate, but exhibits no activity on the boron-deficient plate. This latter observation, of the inability of germanium dioxide to substitute as a "boron" source for *C. fusiformis* was repeated in an experiment in which flax seedlings were grown on nutrient culture in the presence and absence of borate with the addition of dissolved Specpure germanium dioxide at concentrations ranging from 5×10^{-8} to 5×10^{-5}M. Table 2 indicates that germanium dioxide caused no enhancement of growth in the absence of borate, and was toxic at 5×10^{-5}M. This result is contrary to that of Skok (1957), who obtained a significant enhancement of the growth of sunflower seedlings with germanium dioxide in the absence of added borate.

In a further experiment with the diatom, the compounds phenylboronic acid and sodium tetraphenylborate were introduced, and germanium dioxide, boric acid, and sodium tetrafluoroborate re-tested. This experiment showed (Plate 1, Fig. 8) that phenylboronic acid acted as a comparable source of boron to boric acid and that sodium tetraphenylborate was inactive as a source of boron (as was sodium tetrafluoroborate, Plate 1, Fig. 7), but strongly phytotoxic. Further experiments showed that *ortho*-, di-*ortho*-, *meta*-, and *para*methoxy phenylboronic acids all induced some growth on the boron-deficient plates and were not strongly toxic, whilst chloro- or bromo- ring-substituted phenylboronic acids induced very little growth on boron-deficient plates, and were markedly toxic on the plates already containing boron.

IV. Discussion

(*a*) *Boron Requirement of* C. fusiformis

The boron requirement for the growth of unicellular algae had received little attention prior to the work of Lewin (1965), who reviews earlier work. This is partly because boron contamination from borosilicate glassware is normally present (Plate 1, Fig. 1), and partly because the most studied unicellular alga, *Chlorella*, either does not show a clear-cut boron requirement (McIlrath and Skok 1958), or cannot be shown to have any boron requirement (Bowen *et al.* 1965), as has also been shown in this communication.

The magnitude of the boron requirement of *C. fusiformis*, first demonstrated by Lewin (1965), has been investigated. The results indicate (Plate 1, Figs. 1 and 2) that the minimum boron concentration (*c.* 10^{-5}M boric acid or *c.* 0·10 p.p.m. boron) which supports the unrestricted growth of *C. fusiformis* on agar, is greater than that required by tomato or flax seedlings grown in nutrient cultures (Neales 1964*a*). Sea water contains *c.* 4·8 p.p.m. boron (Altman and Dittmer 1964).

(*b*) *Relationship between Chemical Structure and Biological Activity of some Boron Compounds—Evidence for the "Complexing Hypothesis"*

The experiments reported in this paper support the complexing hypothesis for the biological action of borate in *C. fusiformis*. This hypothesis has been described in the Introduction of this paper. The evidence is summarized below.

(1) All compounds that were active in supplying "boron" to this diatom on boron-deficient plates had at least two –OH groups attached to the boron atom. These compounds were boric acid and phenylboronic acid and its derivatives (Plate 1, Figs. 2, 7, and 8). These compounds are also able to complex with diols *in vitro* (Weigel 1963).

Chloro-, bromo-, phenyl-, and phenoxy- substitution in the phenyl ring of phenylboronic acid reduced the activity of these compounds on boron-deficient plates and also increased their toxicity to growth on plates containing boron when compared with phenylboronic acids and its methoxy, ring-substituted derivatives (Plate 1, Fig. 7). The contention that phenylboronic acid and its derivatives are active as sources of boron because they are broken down to phenol and boric acid is unlikely, because phenylboronic acid is stable in aqueous solution and in chromato-

graphic solvents (Neales 1964*b*). Also the growth rates of the diatom on agar medium containing either phenylboronic acid or boric acid were similar.

(2) The compounds sodium tetrafluoroborate and sodium tetraphenylborate were completely inactive as sources of boron for the diatom (Plate 1, Figs. 7 and 8). Aqueous solutions of these compounds, from spectrophotometric evidence, also fail to complex with the disodium salt, catechol-3,5-disulphonic acid (Wildes and Neales, unpublished data) whilst boric acid and phenylboronic acid both complex with this reagent, used by Hiiro (1962) to determine borate in aqueous solution.

Hence it is possible to conclude that, of the boron compounds tested, those which have the ability to complex with diols *in vitro* also show biological activity *in vivo*. However, germanium salts in aqueous solution also complex with organic diols (Weigel 1963), but a solution of Specpure germanium dioxide was unable to replace borate in *C. fusiformis* (Plate 1, Figs. 7 and 8). If the complexing hypothesis is generally valid for diatoms and higher plants, this result must be due to a difference in the solid geometry of the $[B(OH)_4]^-$ and $[Ge(OH)_6]^{2-}$ (or one of its hydroxide derivatives). Weigel (1963) states that the O–O distances in triagonal $B(OH)_3$ and tetrahedral $[B(OH)_4]^-$ are 2·36–2·39 and 2·40–2·44 Å respectively, whilst he deduces that the O–O distance in octahedral $[Ge(OH)_6]^{2-}$ to be approximately 2·64 Å. It is thus possible that the lack of activity of dissolved germanium dioxide as a "boron" source in *C. fusiformis* and flax seedlings (Table 2) is due to the above difference in the arrangement of the –OH groups of $B(OH)_3$ and $[B(OH)_4]^-$ on the one hand and of $[Ge(OH)_6]^{2-}$ on the other. Skok's (1957) demonstration of the incomplete and ephemeral replacement of the born requirement of sunflower seedlings with dissolved Specpure germanium dioxide might be due to the *in vivo* "borate receptor" of the sunflower seedlings being more tolerant of differences in the –OH geometry of the "borate" source, than is the receptor in *C. fusiformis* and flax seedlings.

(*c*) *Alternative Hypothesis to the Complexing Hypothesis*

Alternative to the "complexing hypothesis" is a hypothesis which relates the biological activity of borate to the fact that boric and phenylboronic acid are both Lewis acids. According to Weigel (1963), "This means that boric acid does not act as a proton donor but as a Lewis acid, accepting the electron pair of the base (for example, OH) to form the tetrahedral anion $B^-(OH)_4$". The alternative hypothesis may thus be formalized by stating that "the biological activity of borate may be attributed to the fact that boric acid (and phenylboronic acid) are Lewis acids".

The results of the experiments reported here do not allow a distinction to be made between these two hypotheses. Indirect evidence for it comes from Neales (1960) who demonstrated that a continuous supply of boric acid was necessary for the growth of the bean radicle. From this physiological evidence it can be postulated that each borate ion undergoes a biologically important reaction only once, and does not participate in some reversible cyclic change as do the biologically essential transition metals, e.g. iron and molybdenum. On the "Lewis acid hypothesis",

proposed above, this once-only reaction is postulated to be:

$$\underset{\text{(triangular)}}{B(OH)_3} + OH^- \rightarrow \underset{\text{(tetrahedral)}}{[B(OH)_4]^-}$$

Ranganathan and Rengasamy (1965) have also suggested that the biological necessity of boron may be partially explained on the basis of its electron deficiency, which makes possible its participation in electron-transfer reactions.

V. Acknowledgments

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EXPLANATION OF PLATE 1

Fig. 1.—Cultures grown for 12 days in liquid media in propylene flasks, with varying additions of boric acid, and placed in stoppered glass flasks for photography.

Fig. 2.—Growth after 15 days on agar media to which varying concentrations of boric acid were added. Top row (left to right): 10^{-4}M, 10^{-3}M, 10^{-2}M, 10^{-1}M, and 1M boric acid; bottom row (left to right): 0, 10^{-8}M, 10^{-7}M, 10^{-6}M, and 10^{-5}M boric acid.

Fig. 3.—Growth response, after 15 days on boron-deficient media with increasing agar content, to the addition of 10 μl boric acid solution (= 100 p.p.m. boron). Agar content (from left to right) 1%, 2%, 3%, 5%.

Fig. 4.—As for Figure 3, but with a more concentrated boric acid solution (= 1000 p.p.m. boron).

Fig. 5.—As for Figure 3, but boric acid (0·1 μmole) added in drops of different volumes to boron-deficient 1% agar medium. Volumes used (from left to right): 10 μl, 5 μl, 2 μl, 1 μl.

Fig. 6.—Growth after 7 days on 1% agar media (*a*) made up with Oxoid agar No. 3 and (*b*) made up with Oxoid Ionagar No. 2, batch 57. Top row: boron added to agar medium; bottom row: no boron compound added.

Fig. 7.—Growth after 7 days on media to which boron had been added (top row) and on boron-deficient media (bottom row) as induced by the following compounds: *A*, water; *B*, boric acid; *C*, sodium tetrafluoroborate; *D*, germanium dioxide; *E*, 4-chlorophenylboronic acid; *F*, 4-methoxyphenylboronic acid.

Fig. 8.—Growth after 12 days on boron-deficient media (top row) and on media to which boron had been added (bottom row) as induced by the following compounds: *A*, boric acid; *B*, phenylboronic acid; *C*, sodium tetraphenylborate; *D*, sodium tetrafluoroborate; *E*, germanium dioxide.

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