Induction of Orientation of Bacterial Cellulose Microfibrils by a Novel Terpenoid from *Acetobacter xylinum*

By W. GEOFFREY HAIGH

Biomedical Research Laboratory, Dow Corning Corporation, Midland, Mich. 48640, U.S.A.

and HANS J. FÖRSTER* and KLAUS BIEMANN

Chemistry Department, Massachusetts Institute of Technology, Cambridge, Mass. 02139, U.S.A.

and NEIL H. TATTRIE and J. ROSS COLVIN

Division of Biological Sciences, National Research Council of Canada, Ottawa K1A 0R6, Canada

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1. The bacterium *Acetobacter xylinum* produces extracellular cellulose microfibrils that form a pellicle in the medium enmeshing the bacterial cells. These microfibrils may show some localized alignment, which can be seen as birefringence when the culture is viewed between crossed Polaroid sheets. 2. An increase in birefringence can be induced by the addition of small amounts of certain classes of lipids, particularly sterols, to the cultures. 3. A crude lipid extract from *Acetobacter* cells induced greatly increased birefringence when added to fresh cultures of this organism. 4. When the bacterial lipids were fractionated, most of the activity was recovered in a complex, polar lipid. The lipid is secreted into the medium during growth and is unstable. The non-saponifiable portion of this lipid is shown to be a 1:1 mixture of a saturated and a monounsaturated C₃₅ tetrahydroxy terpene with a hopane ring system in the accompanying paper by Förster *et al.* (1973). The saturated molecule is referred to as tetrahydroxybacteriohopane. 5. Tetrahydroxybacteriohopane is itself capable of inducing birefringence in cultures as is 22-hydroxyhopane, which was also isolated from the non-saponifiable fraction of the total lipids. 6. The mechanism of induction of birefringence (orientation of microfibrils) is not known. This is unlikely to be a specific effect, since all the above compounds are active (intact lipid, tetrahydroxybacteriohopane, 22-hydroxyhopane), as are other classes of lipid. It is suggested, however, that a common mechanism may be involved and that similar compounds may be concerned with control of microfibril alignment in the cells of higher plants.

In most cells of higher plants, the deposition of cellulose microfibrils in the secondary layer of the cell wall is highly ordered. The means by which this orientation is brought about is unknown. Stress owing to turgor pressure has been eliminated as a controlling factor by the studies of Green & Chen (1960), and suggestions of control by internal membrane systems, such as the Golgi apparatus in *Pleurochrysis* (Brown *et al.*, 1969) seem to lack general application.

The two theories that have gained widest support are the control of microfibril deposition by (i) plasma-lemma particles or (ii) microtubules. In support of the first, particles on the outer surface of the plasma-lemma have been observed by some workers to be in an order corresponding to the orientation of microfibrils on the inner surface of the cell wall (Mühlethaler, 1967; Preston & Goodman, 1968; Northcote & Lewis, 1968), but other investigators have been unable to support this (Chafe & Wardrop,

1970). In support of the second, many workers have reported that the direction of microtubules is the same as that of microfibrils in the adjacent cell wall (for review see Newcomb, 1963). However, microtubules have been observed where their orientation does not coincide with that of the cellulose microfibrils (Northcote & Lewis, 1968; Kiermayer, 1970), or coincides only intermittently (Chafe & Wardrop, 1970). In addition, it is difficult to understand how microtubules inside the plasmalemma and apparently not in contact with it (Newcomb, 1963) could control the formation of microfibrils outside the plasmalemma.

Therefore there is no agreement as to whether microfibril orientation is controlled by the plasma-lemma, by the particles on it or by microtubules internal to it. The means by which these organelles could exert control is equally obscure. Induction of microfibril alignment by chemical means has been observed in cultures of bacteria and may yield information relevant to higher plants. Some of the cellulose microfibrils produced in the external

* Present address: Gerichtlich-medizinisches Institut der Universität Zürich, Chemische Abteilung, Zürich, Switzerland.

medium of *Acetobacter* are aligned as bands (Ben-Hayyim & Ohad, 1965), as parts of spherulites (Colvin, 1965), or as thread-like structures (Colvin, 1966). Enhanced orientation can be induced by the addition of small amounts of certain classes of lipids to the medium (Carson *et al.*, 1967). The most-active compounds are sterols. These observations prompted an investigation of the lipids of *Acetobacter xylinum* to determine if any were secreted from the cell and were capable of inducing such alignment. Initial studies showed that a total lipid extract was indeed active in inducing alignment, as was the non-saponifiable fraction of the lipids.

The purpose of the present paper is to describe the isolation, purification and properties of a diffusible complex lipid and its close relatives from *A. xylinum* which are active in inducing microfibril orientation. The most active material was only partially characterized as the intact lipid but a complete description of the non-saponifiable portion of the molecule was obtained.

Materials and Methods

Growth of cultures

Cultures of *A. xylinum* were inoculated, grown and observed under the conditions described previously (Colvin, 1965). Fernbach flasks containing 1 litre of the medium of Hestrin & Schramm (1954) were inoculated with 15 ml of a standard subculture, incubated for 24 h at 30°C and then the contents mixed thoroughly with 5 litres of the same sterile medium. The suspension was then poured into glass Petri dishes (each 15 cm in diameter) to a depth of 0.5 cm. The Petri dishes were supported in a rack so that they could be observed periodically above a 60 W tungsten lamp and between crossed Polaroid films without disturbance. Birefringent areas in the pellicle of the culture appeared as bright regions against a dark background.

Detection of the capacity to induce birefringence (i.e. orientation of cellulose microfibrils)

At all stages of lipid fractionation, each portion was tested for its ability to induce birefringence in the freshly grown pellicle of *A. xylinum* cultures. As shown in previous studies (Colvin, 1965, 1966; Carson *et al.*, 1967) and confirmed in this investigation for the particular lipids involved (see the Results section) birefringence in the pellicle or a part of it implies partial orientation of the cellulose microfibrils within that part. Therefore whenever a compound or fraction is called active or biological activity is mentioned in the succeeding text, this refers to the capacity of the fraction to induce birefringence and mutual orientation of microfibrils during the assay procedure.

Assays for the capacity to induce birefringence were carried out as follows. Immediately after the cultures were poured into the glass Petri plates 1, 2, 5, 10 or 20 drops of a 1% solution of the fraction to be tested in absolute ethanol were placed in the centre of the plate. Triplicates were used for each portion tested. For each plate at each concentration of sample a control plate was used, to which was applied the same number of drops of ethanol. All plates were observed between crossed Polaroid sheets periodically up to 48 h and scored for birefringence on a scale from 0 to 5. This method is qualitative rather than quantitative and the type of birefringence observed differs with different compounds (Carson *et al.*, 1967). The majority of compounds, however, induced the same type of birefringence (Carson *et al.*, 1967, Fig. 2). Because there is a maximum in the relationship of concentration to birefringence for most compounds, all fractions were tested initially over a wide concentration range.

Optical microscopy and electron microscopy

For verification of the association between induced birefringence and the alignment of cellulose microfibrils, the following methods were employed. Birefringence was induced in the bacterial culture pellicles by the appropriate compounds as indicated above. These pellicles and those grown in control cultures were then examined between crossed Polaroid sheets to identify birefringent or non-birefringent areas. Small portions of each type of area were excised by scissors, gently washed free of all water-soluble substances by distilled water and then freeze-dried to remove the water but preserve the original disposition of the surface of the microfibrils. The freeze-dried segments were then rotation-shadowed with gold and examined for orientation of the microfibrils in a Cambridge Stereoscan MK2 instrument at 20 kV.

Lipid isolation

Intact lipid. Pellicles from the cultures of *A. xylinum* grown as described above were extracted by the method of Bligh & Dyer (1959) after chopping the pellicle with scissors. The resultant chloroform layer was evaporated, and the lipid extract was washed by the method of Folch *et al.* (1957). This extract was fractionated on a column (2.2 cm × 55 cm) of DEAE-cellulose prepared as described by Nichols & James (1964). As a routine, batch separations were performed as follows: the neutral lipids were eluted with two column volumes of chloroform, neutral phospholipids with five column volumes of chloroform-methanol (6:4, v/v), and acidic phospholipids with four column volumes of chloroform-methanol (2:1, v/v) saturated with conc. aq. NH₃.

Activity was confined to the acidic phospholipid fraction.

The acidic phospholipid fraction was examined by t.l.c. on plates of silica gel G (1 mm thick). The plates were developed in chloroform-methanol-water (65:20:3, by vol.). The greater part of the activity was associated with a band that migrated with R_F 0.3 and the remainder was confined to the solvent front. The substance of R_F 0.3 was eluted with chloroform-methanol (2:1, v/v) and its homogeneity tested by two-dimensional t.l.c. on silica gel G. The plates were developed first by chloroform-methanol-acetic acid-water (85:15:10:3.6, by vol.) and in the second dimension by chloroform-methanol-water (65:25:4, by vol.). One major spot, which appeared to be more than 90% of the substance (as shown by charring with H_2SO_4), and two minor contaminants were observed. The major component was biologically active, but there was insufficient material in either of the minor spots to test them. After storage of the major component at $-15^\circ C$ in chloroform-methanol (2:1, v/v) for 1 week, three to four additional, closely related, compounds could be observed. Because of the instability of the intact lipid and because the non-saponifiable moiety of the intact lipid was active also, this part of the intact lipid was investigated separately.

Non-saponifiable moiety of the intact lipid. Preliminary studies on the non-saponifiable portion showed that it was unstable, as demonstrated by loss of biological activity during storage. Because examination by i.r. spectroscopy showed an abundance of free hydroxyl groups in this fraction, they were acetylated by the method of Fritz & Schenk (1959). The resultant acetate ester was quite stable and much more soluble in organic solvents, but it had to be saponified to the unstable alcohol to show biological activity. Large-scale preparation of the acetylated material was done by either of the following methods. (i) The total lipids were extracted as described above and then saponified with 3M-methanolic KOH under reflux for 1 h. The non-saponifiable material was extracted with ether, washed with water and the solvent was evaporated. The residue was then acetylated (Fritz & Schenk, 1959). (ii) A more-direct method was to acetylate the total lipid extract by boiling under reflux for 8 h with acetic anhydride-glacial acetic acid (3:2, v/v) (Thompson, 1965). After dilution with water, the lipids were extracted with ether, washed and the solvent removed.

The material obtained from either of the above procedures was fractionated on thin-layer-chromatographic plates developed in light petroleum (b.p. $30-60^\circ C$)-diethyl ether-acetic acid (90:10:1, by vol.). To identify compounds the plates were sprayed with 2,4-dichlorofluorescein (0.1%, w/v, in 95% ethanol) and viewed under u.v. light. Eight bands were detectable. The substance in each of these bands was eluted

with chloroform and tested for biological activity. No compound showed activity as the acetate ester, but when they were saponified to the free alcohols strong activity was found in fraction 2, which migrated with R_F 0.2 as the acetate. Some activity was also observed in the compound from fraction 8 (solvent front) but all others were inactive. The compound from fraction 2 was purified as the acetate by precipitation from chloroform solution with methanol and the resultant white solid was recrystallized twice from a minimum amount of hot methanol. Two-dimensional t.l.c. with light petroleum (b.p. $30-60^\circ C$)-diethyl ether-acetic acid (90:10:1, by vol.) followed by ethyl acetate-light petroleum (b.p. $30-60^\circ C$) (1:3, v/v) showed the crystalline material to be approx. 95% pure (H_2SO_4 charring). It was then recrystallized again from hot methanol. The compound in fraction 8 was eluted from thin-layer-chromatographic plates and, when the solvent was removed, the residue solidified slowly at room temperature to a white waxy mass. This material was recrystallized from chloroform-methanol (1:1, v/v).

Alcohol. During thin-layer-chromatographic fractionation of the total lipids in diethyl ether-benzene (1:39, v/v) the intact active lipid referred to above remained at the origin. A substance with an R_F value between 0.8 and 1.0 under these conditions also showed some biological activity and this compound was subsequently identified as 22-hydroxyhopane. Details of its characterization are given in the accompanying paper by Förster *et al.* (1973), but it is mentioned here because of its biological activity.

Results

Intact lipid

A chloroform solution of the intact lipid was examined by i.r. spectroscopy and the spectrum showed evidence for the following functional groups: free hydroxyl at 3570cm^{-1} ; carbonyl at 1755 and 1720cm^{-1} (probably some ester carbonyl); and a broad band from 1075 to 1100cm^{-1} , possibly a phosphate ester. This lipid was unstable on storage at $-15^\circ C$ as judged by t.l.c. Consequently, characterization was suspended in favour of the isolation and identification of the non-saponifiable part of this molecule. However, the following conclusions could be drawn from the thin-layer- and column-chromatographic mobilities of the compound and its i.r. spectrum: it is a very polar lipid, probably contains an ester phosphate group, a free hydroxyl group and possibly other hydroxyl groups esterified to fatty acid.

To prove that the active lipid was not derived from the nutrient medium, a quantity of the complete medium (Hestrin & Schramm, 1954) equal to that used to produce a batch of cells was extracted. No

trace of a lipid with a similar mobility in t.l.c. was seen. In addition, when *A. xylinum* cultures were grown in the presence of [^{14}C]acetate the active lipid that was subsequently isolated was radioactive, as shown by radioautography of the thin-layer plate. The lipid is thus synthesized by the bacteria and is not simply absorbed or adsorbed from the components of the medium. It was also established that, in a typical culture, 80% of the intact lipid was found on or in the bacterial cells and the remainder was released to the aqueous phase.

Non-saponifiable moiety of the intact lipid

The principal compounds in fraction 2 have been shown to be the tetra-acetates of a C_{35} terpene tetraol (compound VI in the accompanying paper by Förster *et al.*, 1973) and a monounsaturated analogue of the above compound. Small amounts of two higher homologues of the above compounds, each containing an additional CH_2 in the ring portion of the molecule, were also present. It is this group of closely related terpenoid tetraols that form a part of the intact lipid and contribute to the induction of birefringence.

A sample of the pure, unacetylated tetrahydroxybacteriohopane (compound VI, Förster *et al.*, 1973) was oxidized by potassium periodate (Förster *et al.*, 1973) to remove the vicinal hydroxyl groups, the product recrystallized and tested for activity. Within the limits of the test, the oxidized compound was as active for the induction of birefringence as the unoxidized material. This observation suggests that the group of adjacent hydroxyl groups has little, if anything, to do with the mechanism of induction of birefringence.

Alcohol

The structure of the alcohol, which was isolated from the fraction of the total lipids that migrated close to the solvent front, was shown to be 22-hydroxyhopane (Förster *et al.*, 1973). The compound showed activity for the induction of birefringence in *A. xylinum* cultures and because 22-hydroxyhopane contains only one hydroxyl group, this observation is consistent with the above suggestion that groups of hydroxyl groups have little to do with induction of birefringence. The presence of 22-hydroxyhopane as part of an ester in the intact lipid fraction would be consistent with the activity observed for the material from the solvent front (see above).

The white waxy mass, which was crystallized from fraction 8, had an activity that was qualitatively different from that shown by the alcohol from fraction 2 or by 22-hydroxyhopane, both of which

induced the development of a wide-spread, diffuse birefringence. The substance in fraction 8 induced a much more localized birefringence, confined to narrow, branched lines and similar to that produced by cholesteryl oleate (Carson *et al.*, 1967, Fig. 1). The compound was identified by analysis as a hydrocarbon (C 87.9; H 12.1%) and was characterized by mass spectrometry as a pentacyclic triterpene(s) (Förster *et al.*, 1973) of either the lupane or hopane type. In view of the occurrence of 22-hydroxyhopane in the total non-saponifiable-lipid fraction (see above and Förster *et al.*, 1973), which has not been exposed to acidic isolation conditions, it is possible that the compound in fraction 8 was formed by acid-catalysed dehydration of 22-hydroxyhopane under the acidic conditions of acetylation. It may then represent a mixture of hopenes (Fazakerley *et al.*, 1959). Two recent reports mention hopenes in bacteria (DeRosa *et al.*, 1971; Bird *et al.*, 1971) but, since the extraction conditions are not specified, it is possible that these workers may have had 22-hydroxyhopane originally.

Association of birefringence and orientation of cellulose microfibrils

Previous studies by optical microscopy and transmission electron microscopy have shown that birefringence in the pellicles of *A. xylinum* is associated with partial orientation of the component cellulose microfibrils (Colvin, 1965, 1966; Carson *et al.*, 1967). It was considered worthwhile to verify the association, taking advantage of a recently available scanning electron microscope. As an example, Plate 1 shows the birefringence induced in a pellicle of *A. xylinum* by 40 μg of tetrahydroxybacteriohopane that had been oxidized by periodate, together with a photograph of the pellicle grown in a control culture. Plate 2 shows the surfaces of the same specimens as seen by scanning electron microscopy. The cellulose microfibrils in the birefringent portion of the pellicle, which was grown in the presence of the lipid, are partially aligned along a common axis, whereas those in the control pellicle are deposited without any discernible orientation. Staining by Crystal Violet and examination of the pellicle by optical microscopy confirmed that the bacterial cells are without orientation in both the birefringent and non-birefringent portions of the pellicles (Colvin, 1965). Similar results were obtained by using the unoxidized tetrahydroxybacteriohopane, 22-hydroxyhopane or the mixture of hopenes. The degree of activity of all these compounds per μg was comparable and of the same order as for the active cholic acids or cholesterol (Carson *et al.*, 1967). The observations verify the previous assumption that the birefringence induced in the pellicles by the lipid is due to partial orientation of the component microfibrils.

EXPLANATION OF PLATE I

*Comparison of the appearance of (a) a culture of *A. xylinum* grown in a 15 cm Petri dish in the presence of 40 μ g of periodate-oxidized tetrahydroxybacteriohopane and (b) a culture of *A. xylinum* grown under identical conditions except that the terpenoid was absent*

Both photographs were made with the dishes between crossed Polaroid sheets. Note the large area of birefringence induced in the centre of the pellicle that was grown in the presence of the terpenoid. The periphery of the pellicle is not birefringent because in that part of the Petri dish the terpenoid was absent.

EXPLANATION OF PLATE 2

*Comparison of the appearance in the scanning electron microscope of the surface of (a) a birefringent portion of the pellicle of *A. xylinum* grown in the presence of 40 μg of periodate-oxidized tetrahydroxybacteriohopane and (b) a portion of a pellicle grown in the absence of the compound*

Note that in the presence of the terpenoid there is a partial orientation of the direction of deposition of the microfibrils, whereas in the absence of the terpenoid deposition is random. Also note that the microfibrils grown in the presence of the terpenoid tend to be grouped into larger bundles than those from the control culture. Both effects contribute to the observed optical birefringence. The reason for aggregation of the microfibrils into larger bundles by the terpenoid is not known. The bar represents 1 μm in both (a) and (b).

Discussion

Our results show that a complex polar lipid may be isolated from the cells or the medium of cultures of *A. xylinum*. This lipid is synthesized by the cells and has the capacity, when placed on fresh cultures, to induce birefringence (orientation of cellulose microfibrils) in the pellicles of this organism. The lipid may be purified to a limited extent, but when stored at -15°C in chloroform-methanol for 1 week, it changes into three or four closely related compounds. This instability has hindered the characterization of the intact lipid, but its i.r. spectrum indicates that the molecule contains one or more hydroxyl groups, a fatty acid ester and a phosphate group. However, when the intact lipid is hydrolysed by KOH the capacity to induce birefringence is retained in the non-saponifiable portion and the active compound has been shown to be tetrahydroxybacteriohopane or closely related derivatives (Förster *et al.*, 1973). It is a reasonable assumption that the active portion of the intact lipid is also tetrahydroxybacteriohopane (compound VI, Förster *et al.*, 1973). This polyalcohol also loses activity during storage unless the hydroxyl groups are acetylated. The acetyl groups must be removed, however, before activity can be demonstrated again. 22-Hydroxyhopane and its dehydration products may also be isolated from the non-saponifiable portion and are also active in inducing birefringence.

The molecular mechanism by which the intact lipid, tetrahydroxybacteriohopane, 22-hydroxyhopane or the hopenes induce orientation of the cellulose microfibrils in the growing bacterial pellicle is unknown. It is clear, however, that the unusual tetrahydroxy side chain on the terpenoid molecule can play little or no role in the mechanism, because it is absent in the periodate-oxidized product, in 22-hydroxyhopane and in the hopenes, all of which are active. The group of adjacent hydroxyl groups may cause the instability of the intact lipid during storage owing to ester interchange among different positions. Similarly, it is possible that the highly polar side chain of tetrahydroxybacteriohopane may promote molecular association of the terpenoid molecules during storage to yield products that are inactive for inducing birefringence. This speculation cannot be tested until more is known about the molecular mechanism of orientation.

One general aspect of the mechanism of induction is evident. From the present work and previous observations (Ben-Hayyim & Ohad, 1965; Colvin, 1965, 1966; Carson *et al.*, 1967) it is clear that the ability to orient cellulose microfibrils is not confined to one class of compounds. The effect is not a specific one and any proposed mechanism must be consistent with this fact.

The fact that the deposition and alignment of

microfibrils occurs outside the cell membrane of higher-plant cells (Newcomb, 1963) has made it difficult to explain how cytoplasmic organelles could control microfibril orientation. The modification by lipids of the extracellular orientation of cellulose in *A. xylinum* cultures may offer a useful comparison with higher plants and suggests that control by microtubules or other means may be mediated by diffusible lipids. Although it is not yet known how these lipids induce alignment it seems profitable to look for similar chemical species in rapidly growing tissues of higher plants.

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