Silicon
biochemistry

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Silicon biochemistry

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General introduction

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This meeting is centred on one element, silicon, but our aim is to cover a great variety of the activities of silicon which are in one way or another linked to biology. First among these are its links with human biochemistry and medicine. At the end come the links between the minerals, mainly soils, and living systems, including microbial and plant life (Fig. 1). We have, then, a difficult mixture of chemistries to consider. On the one hand we must look at the solid state/solution chemistry of the soil in which, at least to a first approximation, organic matter plays no part, and on the other hand we must look at molecular chemistry in life. The first paper, by Dr Farmer, outlines mineral/solution chemistry. To put silicon chemistry in the general context of biology I shall then give an overview of this subject, from minerals through amorphous materials to biological function, stressing particularly the differences between the silica deposits in biology, biominerals, and the more crystalline minerals of the rocks and soils. One striking feature is the intimate role of aluminium in geological (soil) minerals and in natural waters, in contrast to its virtual absence from biogenic opal. (These silica biominerals differ from manufactured ceramics and amorphous glasses and the distinction is important.)

Undoubtedly the silica of biological minerals is the major part of the biochemistry of silica, representing a turnover close to one-tenth of the biological turnover of carbon. It has had a major impact on sedimentary rocks (Fig. 1). The nature of biological silica mineral is therefore analysed in three separate papers describing different organisms. The silica is far from a uniform material. At this stage of the discussion the ways in which silica and silicates can be introduced into our own bodies through the environment and the food-chain will have been described (see Fig. 1), but nothing in detail will have been said about molecular mechanisms. It is then appropriate that against this lack of knowledge of molecular species Dr Fleming should give his paper which provides a lead to the possible chemistry of silicon from the point of view of the organic chemist. Here new attitudes appear since this chemistry is not restricted by atmospheric oxygen and water. Humans can synthesize a large variety of compounds of silicon not just bound to oxygen atoms, and these are

ACID RAIN

MINERALS ⟶ AQUEOUS ⟶ LOWER ORGANISMS
SOILS   SOLUTION   (BIO-MINERALS)

MAN

INDUSTRY
(MATERIALS)

DRUGS
BIO-MATERIALS
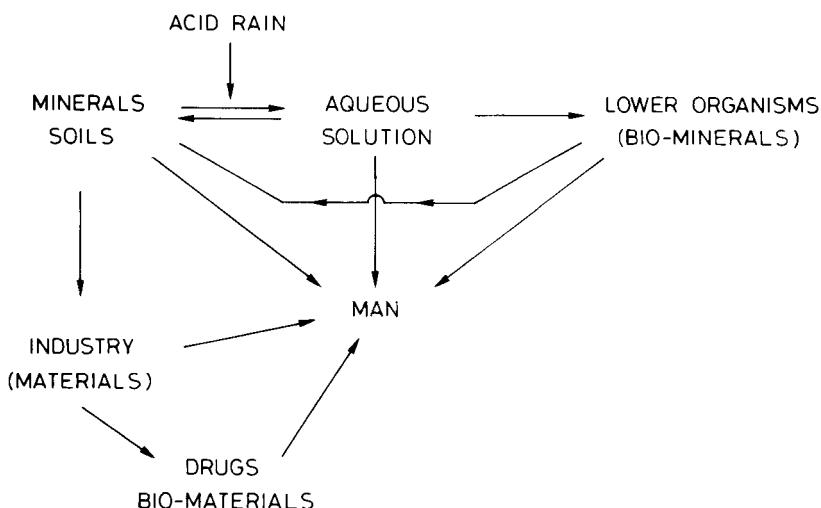
FIG. 1. The various ways in which silicon is introduced into humans.

known to be of great importance in synthetic organic chemistry. Do they exist in biology? Can they be useful to humans and if so in what way—as polymers (silicones), materials, or molecular drugs (Fig. 1)?

Professor Carlisle looks next at the importance of silica in higher animals, i.e. the usefulness of silicon or even its essentiality. Her description will introduce a question not faced up to in the earlier parts of the symposium which described biomineral silica in biological environments, since it is thought that it is *molecular* species that are important in human biochemistry. Such molecules are also necessary in all uptake mechanisms. Another set of molecular interactions are those of silicic acid with metals, as described by Professor Birchall.

Before we expand on the usefulness of any such chemical approach it is important to know not only the way in which biology itself handles the central element, silicon, but also the potential hazards which can be created by its introduction into a living system. The first hazards are the hydrolytic molecular reactions. Si, like Al, can generate OH^- locally while Mg and P do not. The second set of hazards are those of solid silica and silicates. A series of four papers stresses some of them (Fig. 1). It is obvious that to a variety of degrees the introduction of 'solid-state' silica and silicate minerals into any organ of higher animals generates problems. It is extremely important that we control mineral dusts and avoid the introduction of molecules which could give rise to such solid compounds within the body. (Does acid rain represent a new threat through aluminium distribution?) Here the physics of particulate matter is at least as important as the chemistry. While we focus on this problem the molecular species must not be neglected.

Given the risks and known hazards of various forms of silica can we devise a useful drug chemistry based on silicon? It could be that the present-day concentration of effort by the drug industry on carbon-centred chemistry is myopic. We do not have to accept it as axiomatic that organic chemistry is carbon-based. It seems likely that many of the drugs of the future will use other elements and silicon looks a good possibility. On the biomaterials front the last paper introduces many exciting possibilities based on the use of synthetic alkali and alkaline earth metal silicates, often incorporating some phosphate, to devise bone substitutes which are fully biocompatible.

It is hoped that the presentations in the book will be viewed against the background of Fig. 1 so that the biochemistry of silicon is seen in all its fullness. A great deal of chemical knowledge is missing.

Sources and speciation of aluminium and silicon in natural waters*

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Abstract. The aluminosilicate minerals of igneous and metamorphic rocks are mostly unstable in earth-surface weathering conditions. In the tropics and subtropics, they are transformed to stable end-products (crystalline clay minerals, oxides and hydroxides) that largely conserve aluminium and iron. In non-calcareous soils in temperate and boreal climates, aluminium can be markedly mobile, and is precipitated as metastable products that include hydrous aluminosilicates, hydroxyaluminium polymers in or on 2:1 layer silicates, and complexes with soil organic matter. The aluminosilicate precipitates formed at pH <5.5 have structures related to imogolite, a unidimensional crystal in the form of a tube of 2.3 nm outer diameter.

These metastable precipitates, both organic and inorganic, are readily remobilized on further acidification, and can release aluminium into streams if the solutions are not neutralized in the subsoil.

Three classes of soluble aluminium species in natural waters have been distinguished by their rate of reaction with complexing reagents, and their rate of adsorption on cation-exchange columns. These are: (a) unreactive, acid-soluble, Al, (b) labile monomeric Al, and (c) non-liable monomeric Al. Group (b) includes simple inorganic species (e.g. Al^{3+}, $AlOH^{2+}$, AlF^{2+}), and group (c) is thought to include organic complexes.

In contrast, silicon occurs dominantly as $Si(OH)_4$ monomers in natural water. Its metastable precipitates include hydrous aluminosilicates and biogenic opal.

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This paper aims to summarize the factors that control the release of aluminium and silicon from silicate materials, and to trace their fate in the environment. Particular emphasis is placed on metastable weathering products, as these form reservoirs of readily remobilizable aluminium and silicon, which can then be taken up by plants from soil solution, and by animals from stream, river and

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lake waters. These metastable weathering products are widespread in acid soils of the temperate and boreal climatic zones, and of cool moist mountainous regions elsewhere. They arise principally by precipitation of aluminium released by acidic weathering in surface horizons of soils. The general process, termed podzolization, is best examined in podzols, in which dissolution and precipitation of aluminium are clearly separated in different soil horizons. An examination of podzols is further justified by the fact that such soils have been implicated in the release of toxic levels of aluminium into streams and lakes in Scandinavia and North America where speciation studies of the forms of soluble aluminium have been made.

In the tropics and subtropics, weathering is not restricted to the soil horizons, but can extend several metres deep. The typical weathering products of silicate rocks are stable end-products which largely conserve aluminium. With increasing leaching, and so with increasing loss of silica, alkali and alkaline-earth cations, these products are: (a) 2:1 layer silicates (e.g. palygorskite, illite, smectite), (b) 1:1 layer silicates (kaolinite) and (c) aluminium hydroxides (gibbsite, boehmite). Kaolinite, because of its slow rate of equilibration, can play little part in controlling concentrations of Al and Si in soil solutions.

In all climatic zones, weathering releases silicon into solution as orthosilicic acid, which is often the major solute in non-saline waters. The concentration of silicon in various natural waters will be summarized, and some metastable reservoirs of silicon noted.

Podzols and podzolization

The principal factors that control soil formation are the soil parent materials, the site (whether freely or poorly drained), the climate, the vegetation cover and the duration of exposure to weathering. For podzols the typical parent material is derived from relatively unweathered silicate rocks which have been comminuted and distributed over the landscape by fluvioglacial action. The site is necessarily freely draining, at least in the upper soil horizons. A cool moist climate promotes the accumulation of acidic organic residues in the surface horizons. Increasing acidification of the rooting zone favours the growth of conifers and heath vegetation, which, in turn, provide organic residues of lower base content, and so of higher acidifying activity, than the residues of grasses and broad-leaved trees.

The soil processes involved begin with acid attack on soil minerals in the surface horizon by organic and inorganic acids derived from decomposing organic matter. It is these acids that have been responsible, over a few thousand years, for the massive dissolution and leaching that produce a well-developed podzol profile (Fig. 1). Over the last century, however, rain containing sulphuric and nitric acids of industrial origin have contributed to the acidification process, but this still seems to be a small contribution compared to
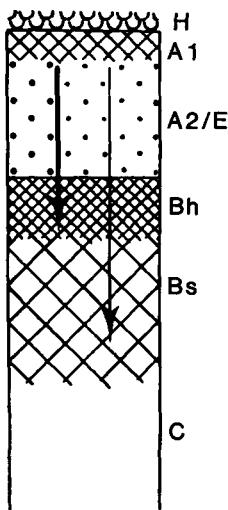
FIG. 1. The podzol profile typically exhibits a surface humus layer (H), a bleached mineral horizon (E), a dark brown humus-rich horizon (Bh), a yellow-red sesquioxide-rich horizon (Bs) and parent material (C). A mixed mineral-organic horizon (A1) may be present. Heavy and light arrows indicate the differential migration of organic matter and aluminium respectively. (Reproduced, with permission, from P. Duchaufour 1979 Pédologie, I. Pédogenèse et classification, Masson, Paris; English edn. Allen & Unwin, London, 1982.)

that arising from decomposing organic matter. Acid rain of pH 4 is less acidic than many podzol surface horizons.

A typical podzol profile (Fig. 1) shows a bleached mineral surface horizon (A2 or E), overlying zones of accumulation of aluminium and iron (B horizons), liberated by acid attack from the E horizon. Within the humus-rich Bh horizon, these elements are precipitated principally in combination with organic matter, while in the sesquioxide-rich Bs horizon they are present, at least in part, as inorganic precipitates. The nature of these precipitates is discussed below. The C horizon is generally considered to represent the unaltered parent material from which the soil developed.

Acid brown soils represent an earlier stage in podzolization, where a distinct E horizon has not developed.

The B horizon precipitates

In the B horizon of podzols, aluminium migrating from the overlying horizons, or generated locally, can be precipitated in three different forms: (a) as insoluble aluminium humates and fulvates, (b) as a crypto-crystalline hydrated

aluminium silicate (imogolite) and a related amorphous form (proto-imogolite allophane), or (c) as a polymeric hydroxyaluminium species which is retained within the interlayers of expanding layer-silicate clays and silts, or adsorbed on their other surfaces.

The relative amounts of the first two forms are approximately estimated by extracting the soil with an acid oxalate solution of pH about 3, which dissolves both forms of aluminium, and with 0.1 M-sodium pyrophosphate, which extracts the aluminium humates and fulvates. Neither reagent extracts the interlayer species (c) effectively, and its amount cannot yet be assessed. Mobilized iron is precipitated as iron humates and fulvates, and as a very poorly organized hydrated iron oxide, ferrihydrite. Again, acid oxalate extracts both forms, and pyrophosphate dissolves the organic precipitates.

In a laboratory study (Farmer & Fraser 1982) of the formation of proto-imogolite allophane and imogolite we showed that hydroxyaluminium cations and silicic acid readily reacted in solutions of pH 3.5–4.5 to form clear, stable solutions containing a non-dialysable hydroxyaluminium silicate species (proto-imogolite) with a limiting Al:Si ratio of around 2.0, when the free silicic acid in solution exceeded about 6 mg SiO_2/l (Fig. 2). When such solutions were
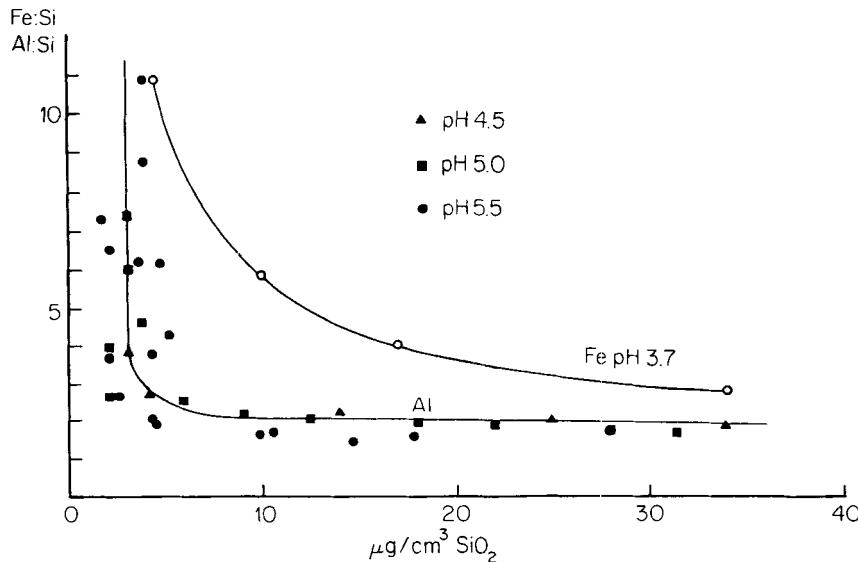
FIG. 2. Variation of dialysable SiO_2 (orthosilicic acid) as a function of Al:Si ratio and Fe:Si ratio in the non-dialysable phase of Al_2O_3–SiO_2–H_2O and Fe_2O_3–SiO_2–H_2O sols. The results indicate the formation of a well-defined aluminium silicate species (proto-imogolite) with a limiting Al:Si ratio of around 2. Silica in excess of this ratio remains in solution as orthosilicic acid. Iron has a much lower affinity for silica than aluminium, and does not form an analogue of proto-imogolite. (From Farmer & Fraser 1982.)
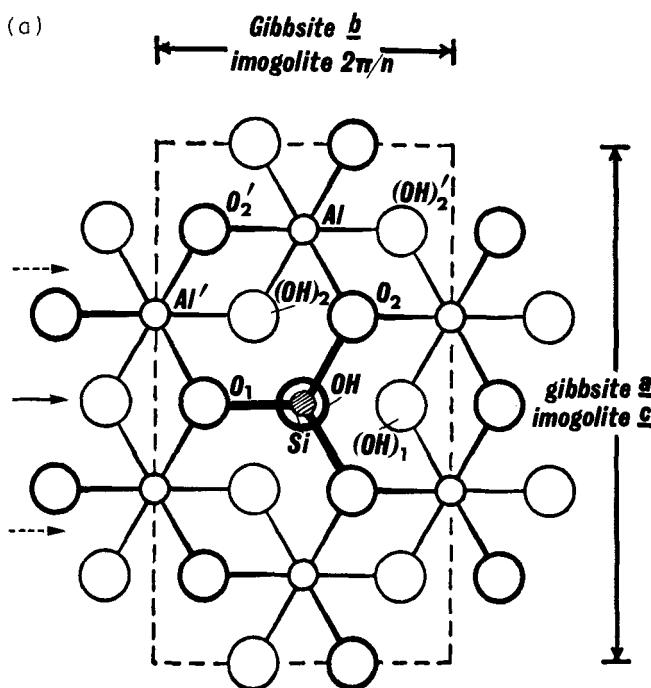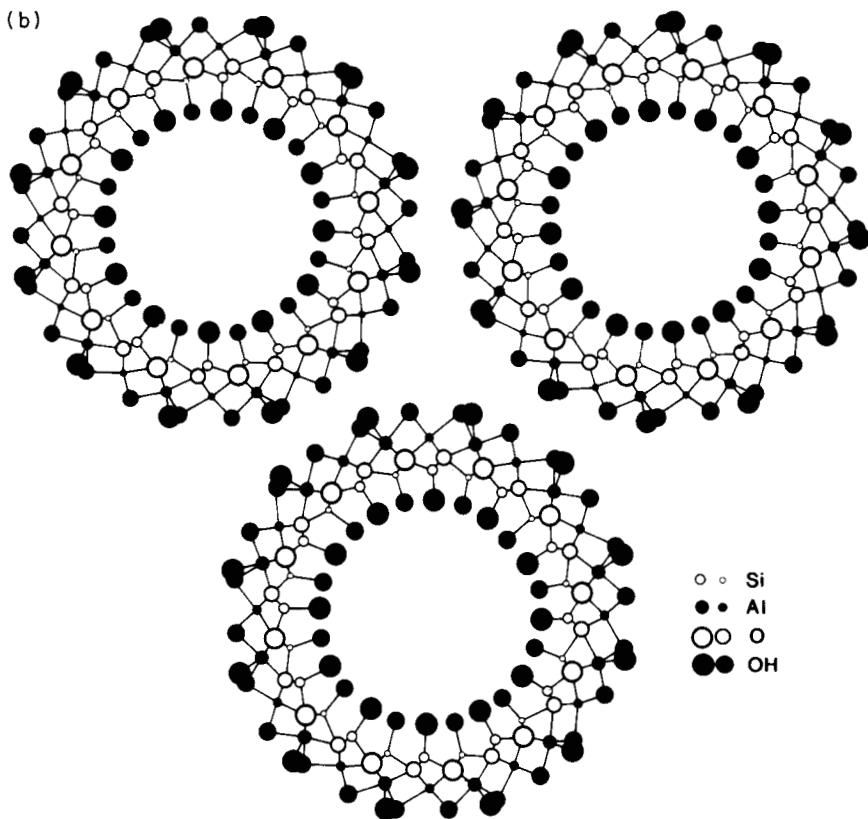
FIG. 3. (a) Mode of attachment of an O_3SiOH group to the face of a gibbsite sheet, causing it to curl to form an imogolite tube. (b) Section of imogolite tubes in hexagonal close packing.

heated they generated imogolite, a unidimensional crystal of tubular morphology. The tube wall can be considered as a single gibbsite ($Al(OH)_3$) sheet, with the inner surface of hydroxyls replaced by orthosilicate (Fig. 3). The ideal composition of imogolite is $(HO)_3Al_2O_3SiOH$. The proto-imogolite precursor is thought to consist of fragments of the imogolite tube wall. Because of the high proportion of edge sites on these fragments, which will not hold silicic acid as strongly as the central sites, proto-imogolite tends to have a higher Al:Si ratio (typically 2.2–3.5) than imogolite. If the proto-imogolite sol is precipitated, the product is proto-imogolite allophane. This material does not reorganize into imogolite, except after redissolution.

For proto-imogolite allophane and imogolite to deposit in the Bs horizon of a podzol, proto-imogolite must be present in the soil solution. Proto-imogolite does not form in solutions in which aluminium is complexed with soil fulvic acid, or with anions that form complexes stronger than lactate. Accordingly, I concluded (Farmer 1982) that such complexes cannot predominate in the soil solution of the Bs horizon.

(b)

Two types of podzol

The relative amounts of the three forms of aluminium precipitated in podzol B horizons vary widely at different sites, depending in a complex way on the soil-forming factors. Two contrasting podzol profiles will be sketched here, one with a B horizon dominated by allophane and imogolite, the other dominated by organic forms of aluminium. These seem to differ markedly in release of aluminium to the environment.

It should be noted that the podzolization process is currently being reassessed (Farmer 1984), and that the views expressed here diverge in some respects from more traditional views (Duchaufour 1982, Theng 1980).

Peaty podzol with thin iron pan (Nethy Valley, Scotland)

The peaty podzol is developed on sands and gravel derived from unweathered granite and acidic schist in the Nethy Valley, Scotland (Farmer et al 1985). The

more readily weatherable minerals, Ca-felspar and biotite, are severely de-
pleted in the E and Bh horizons relative to the Bs and C horizons, and the
Ca-felspar residues are deeply etched in the upper horizons. The acidic attack
effects total dissolution of Al, Fe, and Si within the zone of attack, with no
residue of silica gel. The more resistant minerals that are concentrated in the E
and Bh horizons include quartz, potash felspar, and muscovite. Analytical data
(Fig. 4) and microscopic examination show that aluminium and silica mobilized
from the E and Bh horizons are precipitated in the Bs horizon almost entirely in
inorganic form as imogolite and proto-imogolite allophane, which cement the
relatively unweathered sands. Precipitation is ascribed to the marked increase
in pH to just over 5 in the Bs horizon, from values of 4.2–4.4 (in water) in
overlying mineral horizons. Cementation excludes roots of the pine and heath
vegetation from the Bs horizon, but there is an accumulation of organic matter
in the overlying Bh horizon, from root decomposition and from organic matter
carried in soluble and dispersed forms from the peaty layer overlying the
mineral soil. The Bh horizon in this profile holds aluminium principally in
organic combination (humates and fulvates), although in much lower amounts
than are present in the Bs horizon. Seasonal reducing conditions in the Bh
horizons have removed iron and concentrated it into a thin iron-oxide-rich pan
at the interface of the Bh and Bs horizons. In high rainfall episodes much of the
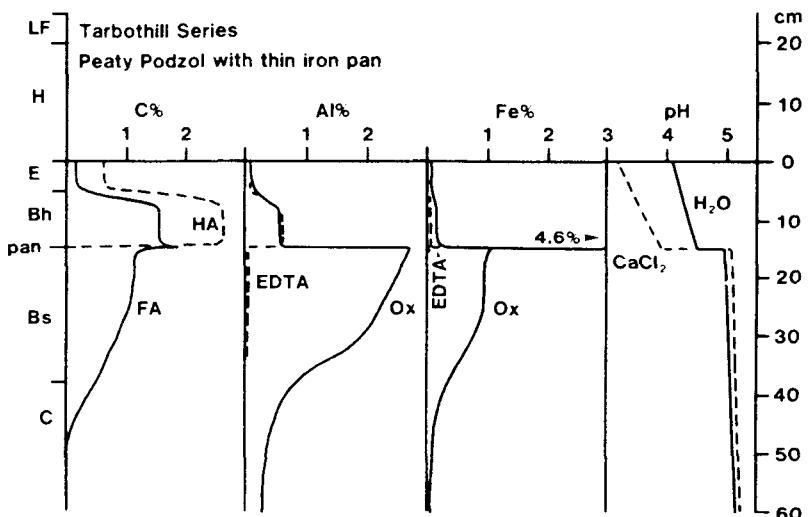
FIG. 4. Distribution of humic (HA) and fulvic (FA) acids, oxalate (Ox) and EDTA-
soluble Al and Fe, and variation of pH in H_2O and $0.01M$-$CaCl_2$ in the mineral horizons
of a Scottish peaty podzol with thin iron pan. Oxalate-soluble allophane and imogolite
are the principal sources of extractable Al in the Bs horizon. (From Farmer et al 1985.)
Symbols as in Fig. 1, except LF = litter.

water drains laterally over iron pans of this type. Where the pan is absent in podzols on this parent material, the interface between the Bh and Bs is more diffuse, and precipitated Fe exceeds Al in the Bh horizon.

Humus-iron podzol; Typic Fragiorthod (New Hampshire, USA)

The humus-iron podzol from New Hampshire (Site 1 of Driscoll et al 1985) is developed on sandy loam or loamy sand derived from pelitic schist (meta-morphosed clayey rock) of sillimanite grade. The analytical data (Fig. 5) show that labile Al is present almost entirely as organic complexes (extracted by pyrophosphate) throughout the B horizons, and even into the C horizon. Proto-imogolite allophane (soluble in oxalate, but not in pyrophosphate) is at most a minor component. This striking difference from the Nethy podzol can be ascribed to the extreme acidity of the profile. The soil pH rises to only 4.0–4.4 in the B horizons, and falls again to 4.2 in the C horizon. Thus the soil, at all depths, is as acidic or more acidic than the Bh horizon of the Nethy profile (pH 4.4), which is a zone of leaching and mineral dissolution. A survey of reported occurrences of allophane in podzols and volcanic ash soils suggests that a soil pH $\geqslant 4.9$ is necessary for its precipitation (Farmer 1984).

The greater acidity of the New Hampshire podzol could be due to several
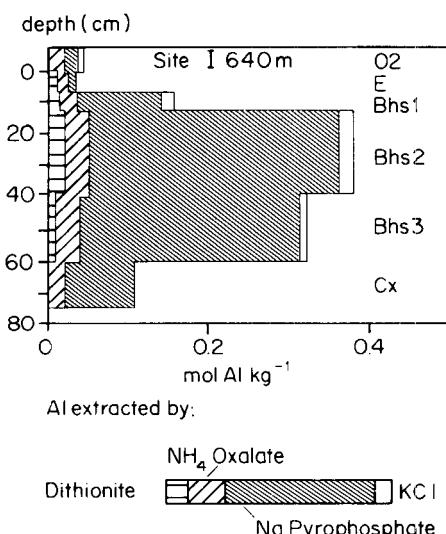
FIG. 5. Distribution of extractable Al in a New Hampshire humus-iron podzol. Pyrophosphate-soluble organic complexes are the dominant sources of extractable Al in all B horizons (Bhs 1–3). (Reproduced from Driscoll et al 1985 Soil Sci Soc Am J 49:437–444 by permission of the Soil Science Society of America.)

TABLE 1. Levels of Al[a] and Si in soil solutions (mg/l) draining from the horizons indicated, and present in adjacent streams or lakes

| | Podzols | | | | | | | | | | Brown soils | | | |
| Horizon | Site 1a | | 1b | | 2a | 2b | 3 | | 4 | | 5 | | 6a | 6b |
	Al	Si	Al	Si	Al	Al	Al	Si	Al	Si	Al	Si	Al	Al
Humus/02	1.0	3.1	0.19	1.4	0.47	0.72	0.73	3.4	0.3	0.6	0.89	0.67	3.3	1.4
A1									1.1	5.0	3.9	2.9		
E/A2	0.65	2.0	0.34	1.5	0.55	1.1	0.68	4.4	1.7	4.0				
Upper B	0.85	2.9	0.28	2.2	0.39	0.81	0.35	3.7			1.1	1.6		
Lower B	0.65	2.2	0.27	3.1	0.04	0.04	0.66	3.6	0.6		0.98	0.71	19	29
Lake/stream	0.71	1.0	0.18	2.8	0.04	0.04	0.02	4.3		3.1				

References for sites: (1a,b) Driscoll et al 1985, Johnson et al 1981; (2a,b) David & Driscoll 1984; (3) Singer et al 1978; (4),(5) Vedy & Bruckert 1982; (6a,b) Nys et al 1981.
[a] Total Al except site 1, where only monomeric Al is reported for soil solutions.

factors. The original sediments from which the rock is derived consisted of pre-weathered residues. The rock contains sulphides which oxidize and release sulphuric acid on exposure to the atmosphere. The substantial clay and silt content provides exchange sites on which Al^{3+}, and H_3O^+, are sorbed on leaching to give strong solid-state acids. Such sites are almost absent in the Nethy profile. The deep rooting of the hardwood forest distributes acidic organic matter from decomposing roots throughout the profile, and is a likely source of the humates that trap aluminium in the B horizon.

A study of the flux of aluminium and silicon in the New Hampshire profile (Table 1, site 1a) shows that the highest levels of Al (1.0 mg/l) and Si (3.1 mg/l) are already present in the solution draining from the humus layer (O2 or H horizon) before entering the mineral soil (E horizon), and that substantial amounts of Al (0.65 mg/l) and Si (2.2 mg/l) are leached from the base of the B horizon. The Al and Si coming from the forest floor is probably principally biocycled via leaf litter although some admixing of minerals with the organic top is possible by windthrow of trees. Driscoll et al (1985) ascribed a drop in Al concentration of 0.2 mg/l within the lower B horizon to precipitation there of an Al-organic complex. However, if biocycling is the source of Al coming from the surface organic layer, then the same amount must be withdrawn from the rooting zone. The soil solution analyses would then reflect only a dynamic steady state, corresponding to a recycling of about 1.0 mg Al/l, and leaching of 0.65 mg Al/l.

The ratio of Al to Si in the leachate from the B horizon (0.3) is consistent with the dissolution of crystalline aluminosilicate minerals, with little loss or gain of Al in organic precipitates within the B horizon. A fractionation of Al in the soil solutions, to be discussed later, indicated that 70–90% of the reactive (monomeric) Al was combined with organic matter at all levels in the profile.

Susceptibility to leaching of aluminium and silicon in podzols

The two profiles examined are of extreme type. The New Hampshire profile has already been extensively leached in depth, but still holds reserves of Al-humates throughout the profile which can liberate Al on further acidification. The Nethy profile has higher reserves of labile aluminium in the form of proto-imogolite allophane in its Bs horizon, but any aluminium mobilized by acid infiltration is likely to be redeposited lower in the Bs horizon.

The Nethy profile, however, holds reserves of aluminium humates above its thin iron pan, which are susceptible to lateral leaching. But an intermediate type of podzol, in which acidification within the Bh/Bs horizons has reached the stage where allophane can no longer be retained, may well be capable of yielding very large fluxes of aluminium and silicon during acidic leaching episodes. The experimental data, reported below, cannot yet test this conjecture.

Mobile aluminium and silicon in podzolized landscapes

Lysimeters inserted in podzols and acid brown soils provided information on the movement of aluminium and silicon now occurring within the soil profile (Table 1). These current processes may well differ from the processes which created the differentiated soil profile now seen, and too few profiles have been studied to reach firm conclusions. Indeed the few soils examined almost all exhibit low pH values (<5) at depth, and have other apparently anomalous features.

Only two of the soils present plausible evidence for the presumed mechanism of podzol formation, i.e. dissolution of aluminium in the upper mineral horizons, and precipitation in the B horizon: these are a podzol (4) and a brown earth (5), both on sandstone in the Vosges (France). These also show a deposition of silicon in the B horizons, indicating the precipitation of allophane. The North American podzols (sites 1–3) commonly show high levels of Al in water draining from the humus layer overlying the mineral soil, with minor additions and losses within the mineral horizons. All the soils show loss of Al in drainage water at the bottom of the B horizon. The highest losses occur not in a podzol but in an acid brown soil developed on acidic silts in the Ardennes, with losses of 19 mg/l under hardwood (site 6a) and 29 mg/l under conifer (6b).

It is a striking fact that, in areas where acid rain of pH 4 has been implicated in the leaching of Al into streams (sites 1 and 2), the levels of Al and Si draining from the B horizons are little or no higher than those from a Washington soil (3) receiving precipitation of pH 5, and the Al is much less than that from the Ardennes soil (6), also with rain of pH 5. In the last example, the acidity is developed within the soil.

Little Al enters streams and lakes when water draining from the B horizon is further neutralized and filtered through deeper subsoil horizons (Table 1, sites 2 and 3). At the New Hampshire site (1), water draining from the B horizon can be diverted laterally over a dense layer of low permeability (the fragipan, labelled Cx in Fig. 5), and enter streams without further neutralization. Johnson et al (1981) and Driscoll et al (1984) have fractionated the soluble aluminium present in such stream waters into three species: (a) acid-soluble Al, (b) labile monomeric species, retained by a cation exchange resin, and (c) non-labile monomeric species, not retained by the cation exchange resin. The two monomeric species were distinguished by rapid reaction with complexing reagents. Johnson et al (1981) and Driscoll et al (1984) consider the labile monomers to be inorganic (e.g. Al^{3+}, $AlOH^{2+}$, AlF^{2+},) and the non-labile monomers to be organic complexes. They did not examine the behaviour of proto-imogolite species: these, if present, would possibly be included with the acid-soluble Al, which is thought to include polymeric hydroxyaluminium cations and non-reactive organic complexes, or with non-labile monomers.

The relative amounts of the three species varied widely through the year (Fig. 6). Low pH and high levels of inorganic aluminium were observed after high rainfall and snowmelt. Low flow conditions produced high pH values and very
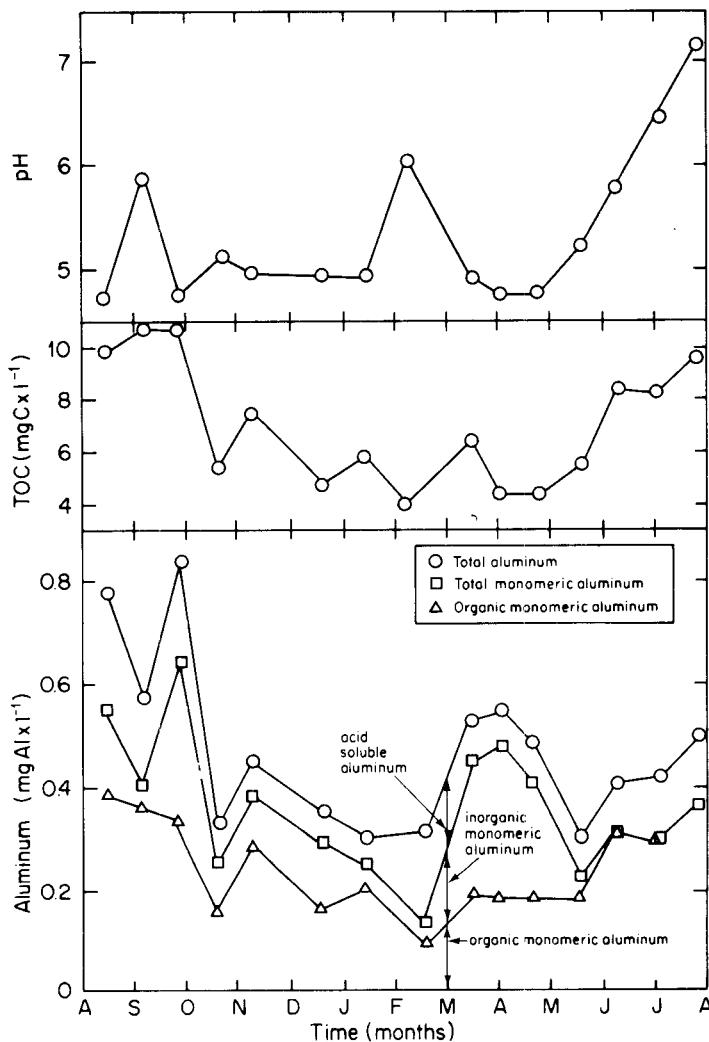
FIG. 6. Changes in pH, total organic carbon (TOC) and aluminium forms for Canachagala Creek (New Hampshire) from August 1977 to August 1978. Low pH and high levels of inorganic aluminium were observed after rainfall (August 15, September 27) and during snowmelt (March 15, April 2, April 23). Low flow conditions produced higher pH values and lower inorganic aluminium values. TOC and organic aluminium were generally high in the autumn and summer, and low in winter and spring. (From Driscoll et al, with permission of Butterworths.)

low inorganic aluminium, but increased amounts of organic aluminium. Acid-soluble aluminium did not vary greatly through the year.

Inorganic monomeric aluminium was further assigned to free Al^{3+} (aquo), $AlOH^{2+}$, $Al(OH)^{+}_{2}$, fluoride and sulphate complexes. Fluoride complexes were commonly the dominant species, but in acidic leaching episodes during snowmelt, Al^{3+} and hydroxyaluminium cations were dominant, as rapid passage of water through the profile reduced fluoride levels. The source of these species must be the Al-humate compounds in surface organic horizons and Bh horizons, as these are the dominant labile forms of aluminium in this profile.

The amounts of inorganic aluminium in these stream waters has been commonly found to suggest equilibrium with the aluminium hydroxide, gibbsite, although this mineral was not detected in the soils. The levels of silicon in the soil solutions and streams (~3 mg Si/l) would, however, favour formation of imogolite or proto-imogolite allophane, rather than gibbsite, in equilibrium with almost the same level of monomeric inorganic Al species. Proto-imogolite allophane is a likely form for deposition of aluminium when the soil solutions are neutralized in deeper aquifers. Such precipitates have been recorded in New Zealand (Farmer 1984).

The amounts of aluminium in acidic streams in Northern USA (<1 mg/l) are not as high as some found elsewhere. In Norway, total monomeric Al concentrations of 4–10 mg/l have been reported in streams of pH 4–4.3 and ~2 mg/l in lake water of pH 4.7 (Y.–H. Lee, unpublished paper, Workshop on Aluminium Speciation, Royal Society, August 1984). Water supplies in the UK have been reported to contain up to 28 mg/l, but it is not clear to what extent this is due to treatment of the water with aluminium sulphate.

Other labile aluminium pools

In addition to the organic and inorganic precipitates of northern podzols, two other forms of labile natural aluminium precipitate are of interest or significance. In tropical and temperate podzols on coastal sands, massive aluminium humate cementation occurs where surface organic-rich solutions meet groundwater (Farmer et al 1983). This so-called coffee-rock often separates overlying 'black water' from underlying 'white water'. These humate precipitates persist for many thousands of years in near-neutral conditions, but can be remobilized by acidic leaching. Acidic leaching in Florida was found to mobilize aluminium from Al-humates when the pH fell below five. This seems to be a crucial pH for mobilizing Al from either allophane or organic matter.

In New Zealand, a spring of pH 5 containing 0.14 mg Al/l and 15 mg Si/l deposits a silica-rich allphane (Al:Si~1.5) when its pH rises to 6.0–7.0 on losing CO_2. This finding suggests that CO_2 may solubilize Al in some way. The allophane differs in structure from proto-imogolite allophane, as it is formed at

higher pH and incorporates some tetrahedrally coordinated aluminium (Farmer et al 1979).

The use of reactive aluminium hydroxide gels as antacid pharmaceuticals (Serna et al 1983) seems likely to produce much higher concentrations of potentially absorbable aluminium in the stomach than any natural source of aluminium.

Silicon in natural waters

The chemistry and geochemistry of silica and silicic acid has been well reviewed by Iler (1979) and Aston (1983), and the dynamics of silicon in soils is thoroughly discussed in a valuable text on *Minerals in Soil Environments* (Dixon & Weed 1977). Silica is often the principal solute in natural fresh waters, where it occurs entirely as monosilicic acid. Typical concentrations of silicon are shown in Table 2, and are compared with the solubility of various forms of silica in Fig. 7. These concentrations are usually greater than the solubility of quartz, and always less than the solubility of a pure, freshly precipitated, silica gel. Quartz, because of the inertia of its dissolution, plays no significant role in determining silicon concentrations in surface water. Ultimately, all silicic acid in solution has derived from the weathering of silicate rocks, and, where a stable dynamic equilibrium has been established, the rate of export of silicon in streams and rivers must correspond to the rate of release of silicon from minerals. There are, however, several possible intermediate reservoirs of different lability that silicon may accumulate in. In climates with a marked dry season, silica may deposit as opal, which will be only slowly redissolved (Fig. 7). Much silica is taken up by plants and returned to the soil as phytoliths, which can make up a substantial proportion of the silt fraction of a surface soil. Phytoliths appear to differ widely in solubility. In the B horizon of

TABLE 2.   Concentration of Si in some natural waters[a]

	Si (mg/l)
Soil solution	1–40
Streams	0.8–15
Groundwater	3.5–28
Lake Erie	0.56
Soda Lake	44
Sea water, bulk	1–7
surface	0.0001–0.2

[a] Data from Bache 1983, Stumm & Morgan 1981, Iler 1979, Dixon & Weed 1977.
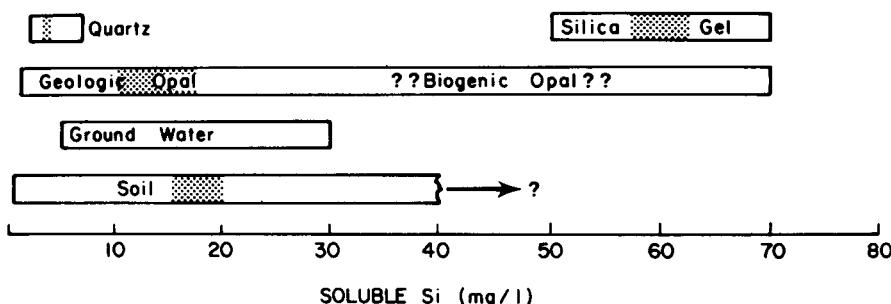
FIG. 7. Relationship between the concentration of Si commonly found in soils and groundwaters, and the solubility of quartz, silica gel, and opal. Stippled areas are commonly reported ranges. (Reproduced from Wilding et al 1977, in 'Minerals in soil environments' p 471–552 by permission of the Soil Science Society of America.)

podzolized soils, proto-imogolite allophane is an important labile reservoir of silicon, which is readily redissolved by complexing acids. The amount of silicon in this form is commonly assessed by extraction with an acid oxalate solution of pH about 3. This extractant also dissolves silicon from other allophanes, such as those formed by the weathering of volcanic glass.

In the tropics and subtropics, silicon released by weathering is partly incorporated into the very stable weathering product, kaolinite, and into smectites, which are stable where leaching of silica and basic cations are restricted.

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DISCUSSION

Williams: The problem of acid rain clearly involves an interaction going from human activity back to the release of elements from minerals in the soil. I didn't stress that in my diagram (Fig. 1, p 2) because we will come to the discussion of aluminium and aluminosilicates later on. Whenever we discuss solubilization of silica we are almost invariably forced to discuss the solubilization and the nature of aluminium species.

Werner: Are organic acids essential for producing imogolite? What other ions can move across the imogolite tube, and do other ions accumulate in this tube?

Farmer: Imogolite is a remarkably pure alumino-silicate. Unlike other silicate clays it has essentially no cation exchange capacity. It can carry a positive charge so that it can be dispersed in acid but not in alkaline solution, making it different from the layer silicates, which have a negative charge. Its small pH-dependent positive charge allows it to trap anions. It can absorb phosphate and organic matter but its capacity for absorption is not great.

There is no evidence yet that there is anything in the middle of the tubes. The less well organized material, proto-imogolite allophane, has a lot of broken edges, and so has a much higher capacity for anion adsorption.

Aluminium has to be brought into solution for imogolite to form, and in soil the principal acidifying agent is organic matter. The organic acids attack primary and secondary minerals to bring aluminium into solution. Most people consider that aluminium moves and precipitates as organic complexes in podzols. I have been arguing that the presence of imogolite indicates that there must be free uncomplexed aluminium in solution to combine with silicic acid in this way. From laboratory experiments we know that quite small traces of complexing acids will prevent crystallization of the imogolite tube. The infrared spectrum of the product shows that imogolite structures are present but the tube hasn't formed when the molar ratio of aluminium to citrate is 20:1. Organic acids impede formation of the tube but they are essential for getting aluminium into solution. Presumably, the complexing organic acids are decomposed by microbial action.

Hench: Have concentrations of soluble silicon been measured at the sea bed? Do the concentrations there reach equilibrium values?

Farmer: In the deep oceans soluble silicon reaches about 7 p.p.m. at the most. At that level it has not yet reached equilibrium. There is a flux of silicon from the sediment back into the ocean and up to the surface. Most of the diatomaceous silica dissolves before it reaches the bottom of the deep ocean. In the sediment itself, amorphous silica transforms into a disordered cristobalite, and finally finishes up as quartz (Aston 1983).

Volcani: Lanning & Eleuterius (1985) have just published a very interesting paper on silica in plants growing in coastal areas of Mississippi (USA). Among the variety of plants which consistently showed the presence of opal a single plant showed the presence of α-quartz. Is this kind of quartz a contamination or can opaline be converted to quartz biologically?

Farmer: It would be very surprising if quartz was formed in opal phytoliths.

Volcani: Is it thermodynamically possible?

Farmer: Yes. Quartz is thermodynamically stable relative to opal, but the transformation is desperately slow.

Volcani: I envisage that these processes are biologically most unlikely.

Williams: It is extremely improbable that the observation about quartz is correct—but it just might be correct.

Volcani: There were similar incorrect observations a number of years ago on the presence of quartz in diatom frustules. Water or soil was contaminating the frustule but there was no genuine quartz in the diatom.

Dobbie: The solubility of silicon in water is extremely puzzling. You said that in stream water and groundwater the concentration of silicon was fairly constant, but we found a marked difference in drinking water in different parts of Great Britain. We measured the amount of soluble silicon by atomic absorption spectroscopy and found concentrations ranging from 7 μmol/l in Irvine to 180 μmol/l in the London area. In the north and west of the country the water is treated with alum but that isn't often done in the south.

Farmer: Putting alum into the water would certainly take out silicon, as well as organic matter.

Dobbie: I thought the effect was due to the acidity of the tributary streams in the north-west.

Farmer: Acidity has no effect on silica solubility, which is independent of pH below 9.

Williams: Did the water you analysed contain soluble silicon rather than dispersed colloidal silicon, Dr Dobbie?

Dobbie: Yes. We measured soluble silicon in samples of tap water.

Farmer: Your lowest figure of 7 μmol/1 works out at 0.2 mg/l and the highest figure of 180 μmol/l at about 5 mg/l. None of the figures I have seen were lower than 0.8 mg/l. Unpublished observations by B.F.L. Smith and B.D. Mitchell (Macaulay Institute) show that, in soils developed on various types of parent rock in North-East Scotland, the concentrations of silicon in the soil solution, sampled over a year, varied from 1.0 to 5.0 mg/l in the surface soil (10–15 cm depth), increasing to 1.4–7.0 mg/l at about 40 cm depth. Streams draining soils derived from readily weatherable basic and ultrabasic igneous rocks contained 7–10 mg Si/l, whereas those draining soils derived from slate, schists and granites contained 3.0–5.0 mg Si/l.

Aluminium concentrations were more variable. The highest concentrations in the soil solution were found in acid soils derived from schists and granite, with concentrations mostly in the range 0.6–1.5 mg/l, but streams draining these soils contained only 0.01–0.3 mg/l. Streams draining soils derived from other parent rocks generally contained less than 0.01 mg/l.

According to a report in *New Scientist* (25 April, 1985, p 7), 20% of drinking-water samples in Yorkshire contained more than 0.2 mg Al/l. Concentrations up to 28 mg/l have been reported. These very high aluminium concentrations may arise from the addition of alum during water treatment.

Dobbie: Aluminium toxicity and its interaction with silicon toxicity are probably going to be discussed later. There has been very little interest in it in the medical world and it is very difficult to get any data on the silicon content of drinking water. This became a problem in renal dialysis in places where a lot of

alum was used, leading to dialysis dementia. We didn't understand what was happening and wondered whether it was due to the acidity of the water in the north and west of the country.

Espie: The North-West cannot really be regarded as a single area. The water for South Manchester, from Peak District reservoirs, has a high peat content and is therefore treated with alum, but in the northern half of that area the water comes from the Lake District and doesn't need to be treated with alum. Incidences of aluminium-related osteomalacia can be correlated with this distribution.

Birchall: As regards the mineral content of water, there is a split diagonally across Great Britain, from the north-west to the south-east, which roughly follows the age of the rocks, with the older, weathered, rocks to the north giving low mineral contents.

Volcani: The assay procedures for silicon (i.e. silicate ion) and for silica (polymerized species) is very important and causes a lot of confusion. One way to describe these species is as molybdate-reactive or molybdate non-reactive, respectively, but that is again an incomplete way of assaying it. We found that it is necessary to carry out two determinations: (1) in an untreated sample for silicate ion; (2) in a sample pretreated with 2 M-NaOH in boiling water for 15 minutes, to hydrolyse the polysilicate to monomeric silicate. This determines whether the polysilicate is rereversible to monosilicate.

Farmer: Surely polysilicic acid is unlikely in natural waters?

Volcani: If you are dealing with acidic water you may get some polymerized species of silicates. If the water has a pH of 7.8 I don't think that would be the case.

Farmer: According to Iler (1979), silicic acid will not polymerize at concentrations below 50 mg Si/l.

Volcani: We have evidence of polymerization in the solutions we are making artificially for culture media.

Mann: How much of the soluble silicon in soil is in the form of complexes? If there are complexes, what types are present?

Farmer: It is usually assumed that all the silicon is present as monosilicic acid. There is no convincing evidence for natural organic complexes. In acidic waters, an aluminosilicate complex (proto-imogolite) can be present, but this can only involve a small proportion of the total silicon in solution, as aluminium is always present in much smaller proportions than silicon.

Williams: One problem is how biology avoids aluminium for a large part of the time. It seems to do this with great skill, yet it seems to take up silicon at the same time.

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Introduction to silicon chemistry and biochemistry

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Abstract. An outline of the chemistry of silicon is given, stressing the reactions in water. In biological systems the metabolism of silicon is little known but much silica is deposited in a variety of amorphous forms. The differences between this biological silica and mineral silicas and silicates, which can be health hazards, are indicated. However both manufactured mineral phase and molecules containing silica can be valuable in medicine.

1986 Silicon biochemistry. Wiley, Chichester (Ciba Foundation Symposium 121) p24–39

The silicon atom in biology is almost certainly always bound to oxygen atoms. This assumption apparently provides a close limitation on silicon chemistry but it has to be viewed against the background of two further features: (1) the ability of silicon to form bonds of variable bond angle and (2) its ability to form four, (five) and six coordination compounds. Given these variations and the possible changes in the oxygen atom from oxide, O^{2-}, to hydroxide, OH^-, or to a variety of alcohol, enol or phenol oxygens as anions or neutral molecules, a bewildering variety of compounds with an even greater variety of stereo-chemistries becomes possible (Iler 1979). The questions arise: which combinations have evolved in biology, and for what functional purposes?
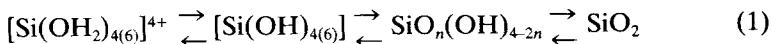
The silicon atom outside biology has some additional possible chemistries. Through high-temperature processes silica can form as quartz, SiO_2, or in various forms of silicate. Notable amongst these, if we keep in mind the association with biology, are the clay or soil minerals which are essentially and obviously in close association with most of plant life. On the other hand, using non-aqueous chemistry, research workers have developed compounds containing Si–N and Si–C bonds as well as combinations with later elements than oxygen in the periodic table, e.g. Si–F, Si–Cl. Just as some of the silicate minerals are harmful whereas others have been suggested for medical use, so some of the non-mineral chemistry of silicon has produced harmful substances while other parts of it have produced potentially therapeutic drugs. The second

question concerns the uses and abuses of silicon compounds and their chemical characteristics.

Silicon, has, then, a rich oxygen chemistry. In this paper I can introduce only salient features. Other contributors will deal with specific issues in detail.

Silicon–water chemistry

Silicon–water chemistry is essentially the chemistry of hydrolysis. In essence, hydrolysis can be postulated to start from an ideal hydrated Si^{4+} cation in water and it then extends through hydroxylated and oxygen species to the ultimate SiO_2 which is not quartz but an amorphous cross-linked polymer:

$$[Si(OH_2)_{4(6)}]^{4+} \rightleftharpoons [Si(OH)_{4(6)}] \rightleftharpoons SiO_n(OH)_{4-2n} \rightleftharpoons SiO_2 \qquad (1)$$

The silicon atom is assumed to be four-coordinate only after precipitation or condensation. Some of the species will be anions since the $Si(OH)$ group is acidic. For the most part we are not concerned with very acidic and very basic solutions, so $Si(OH)_4$ is the conventional starting point. It would appear to be common in all water (fresh and sea), and is stable as a saturated dilute solution of about $10^{-4}M$. Attempts to increase the concentration of $Si(OH)_4$ lead to precipitation of a solid phase in which it condenses to form silica gels, $SiO_nOH_{(4-2n)}$ where n varies from close to 0 to close to 2. It is the insolubility of condensates of $Si(OH)_4$ and their high pK_a values which lead to the prior precipitation of hydrated SiO_2 rather than the formation of insoluble calcium or magnesium salts.

The behaviour of phosphorus acids is very different. They readily give anions
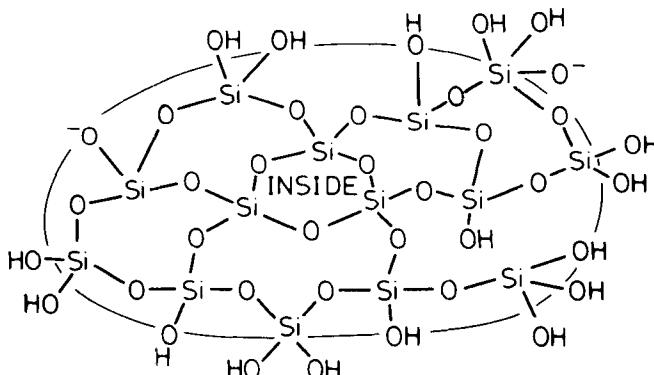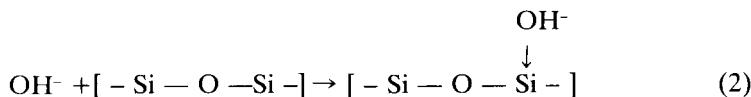
FIG. 1. Schematic illustration of the nature of amorphous hydrated SiO_2. Notice the different numbers of OH groups on different silicons, the absence of structural repeat, and the few surface charges.

and insoluble salts but not cross-linked polymers since they have only two –OH groups, at most, between pH 3 and pH 6. Thus the biochemistry of silicon is most unlike that of phosphorus. The SiO_2 phase which forms is likely to be inhomogeneous and increasingly dehydrated as the centre of the precipitated particle is reached (Fig. 1). This means that the particle formed is invariably non-stoichiometric. It will give no X-ray diffraction pattern or lattice image in the electron microscope (Mann & Williams 1982). Considerable energy is required to drive off all the water since perfect interlocking tetrahedra must form to give quartz. The kinetic barrier to such a change is so large that it is not known to occur in biology. Moreover the reverse reaction of the hydration of quartz is extremely slow and biological systems can hardly attack such a mineral.
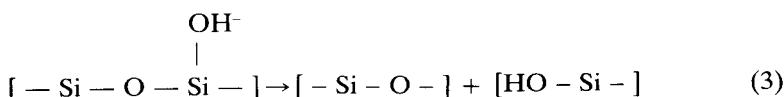
In summary there are two realms of solid silica chemistry. The first is the amorphous state represented by silica gels of all kinds. Here the chemistry is of kinetically labile precipitates. The second is of the crystalline state of highly stable minerals that only change over years. The extremes are well known in chemistry but we also know that there are intermediate conditions, since we can produce a somewhat stable amorphous mineral, opal, by heating amorphous silica, and we are all familiar with the ranges of soil minerals. It is the differences in kinetics which make for the gross differences in response of biological systems which are in contact with silica-based solids, amorphous and crystalline.

Kinetics of SiO(OH) matrices

There is clear evidence that the oxygen of the OH groups on Si in solution exchange and that the exchange is fast. It is promoted by alkali and probably proceeds by an associative mechanism. The bridging oxygen atoms do not exchange so easily and can become totally protected from hydrolysis in silicone polymers. In the more solid but open networks of amorphous SiO_2 the initial step is presumably

$$OH^- + [-Si-O-Si-] \rightarrow [-Si-O-\overset{\displaystyle OH^-}{\underset{\displaystyle \downarrow}{Si}}-] \qquad (2)$$

to be followed by

$$[-Si-O-\overset{\displaystyle OH^-}{\underset{\displaystyle |}{Si}}-] \rightarrow [-Si-O-] + [HO-Si-] \qquad (3)$$

Polymerization–depolymerization is relatively rapid from the gel silica yet the
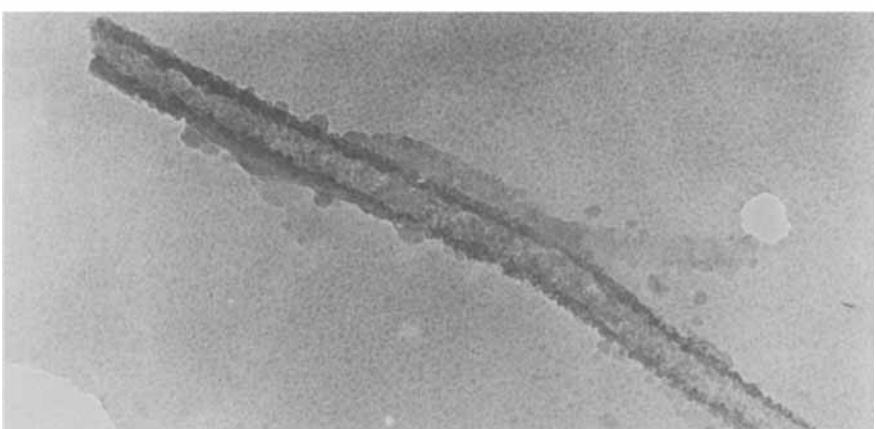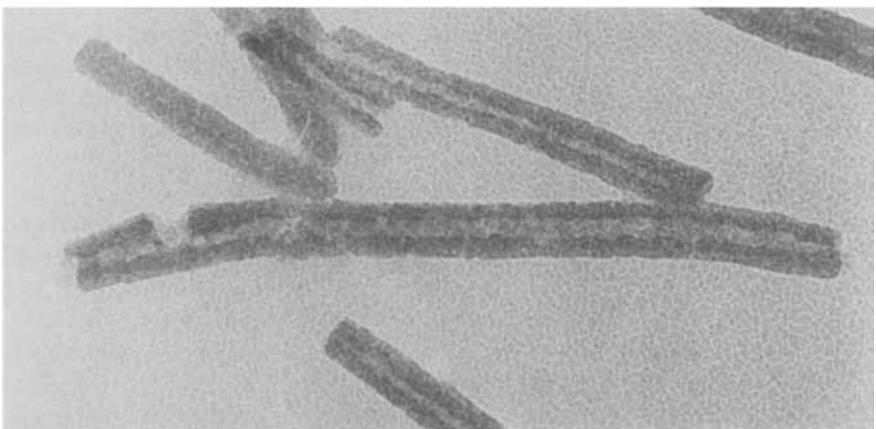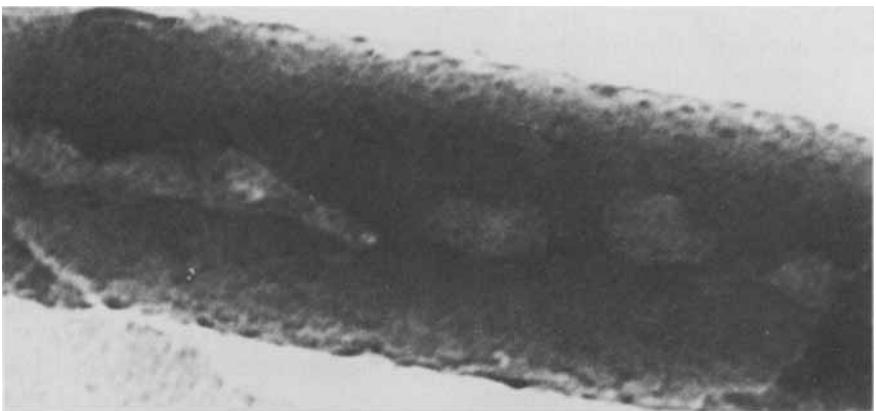
FIG. 2. The curved rods of a choanoflagellate showing the hollowed centre from which SiO₂ dissolves. Top × 620 000; centre and bottom × 145 000.

removal of $Si(OH)_4$ units from quartz is a very slow process. Presumably it is the very regular tetrahedra of quartz which prevent access of OH^- and H_2O to the silicon, which stops any reaction, while amorphous hydrated silicas easily exchange water.

The protonation–deprotonation reactions of $Si(OH)_4$ are very rapid and there seems to be no kinetic barrier of interest. Given that an SiO.OH surface carries some negative charge, the surface of a silica gel must also be a useful proton conductor surface and a good cation exchange bed. It is curious that the silica in biology is almost free from cations of any kind, though we detected association with some potassium in the silica of plants (Grime et al 1985).

If there are some six coordinate species in hydrated silica in parallel with SiF_6^{2-} or $[Si(catecholate)_3]^{2-}$ then the mechanism of hydrolysis of these centres is likely to be by dissociation of one ligand to give a five-coordinate unit. The reaction is likely to be rapid. The surfaces of silica gel may contain some species of this kind. The overall impression of hydrated SiO_2, especially its surface, is that it is labile, acidic, and maybe catalytic, and therefore we need to know how it is stabilized and protected by polymers in biology. NMR methods give no evidence for higher coordination numbers than four in most silica gels but the nature of the surfaces is unknown (Mann et al 1983).

It will become apparent in this symposium that biological systems can control the degrees of polymerization (hydration) of amorphous $Si(OH)_4$. For example, in plants there are many gross forms of silica but all appear to be made from roughly spherical (10 nm) particles of $SiO_n(OH)_{4-2n}$ (Perry et al 1984). On the other hand the rods of choanoflagellates would seem to be made from a much larger continuous matrix of $SiO_n(OH)_{4-2n}$. Some of these types of 'opal' dissolve quite readily. Mann & Williams (1982) showed that the choanoflagellate rods dissolved from the inside, indicating that they are a layered cylindrical structure in which the silica at the outer edge is either protected by an organic layer (see below) or is less hydrated than the innermost region (Fig. 2). Plant silicas do not appear to be so easily dissolved and they have a variable hydration. We shall return to these points later but they bring out one way in which amorphous silica can be protected by polymers.

Association of amorphous silica and organic materials

A major feature of the highly hydroxylated surface of silica gel is the ability of polar organic molecules to stick to it (Iler 1979). The use of silica gels in chromatography is an obvious example. There is then the immediate question of the selectivity of this interaction. Have biological systems devised special hydroxylated surfaces, polysaccharides, for combination with silica gel? Do they stimulate precipitation and control the chemical character of the gel? Alternatively they may be synthesized as preformed containers in which silica gel is deposited and partially stabilized. As I suggested above, the kinetic

properties of silica gel, including its easy dissolution, depend on the exposed hydrated surface. Once this surface is coated by a hydrogen bond network of, say, polysaccharide, the silica gel is a stable unit. The prevalence of 10 nm particles of silica in plants and of silicon rods in protozoa (Leadbeater 1979) suggests that there are special ways of stabilizing such units with polysaccharides.

I have not referred to the covalent condensation of silica hydroxyl groups with organic –OH groups. I expect this topic to be discussed at length later. The question of kinetic control of silica through such chemistry is wide open. Indeed it has been frequently postulated that organic hydroxyl groups, especially of phenols, condense with Si(OH) in biology and that these may play a special role in uptake and transport. There is little evidence that these condensates occur, to my knowledge, so the composites of silica gels and polysaccharides may well just be hydrogen-bonded. Considering the selectivity of association in such materials as cartilage there is still much work to be done before we understand these associations (see Sposito 1984 and Fig. 3).
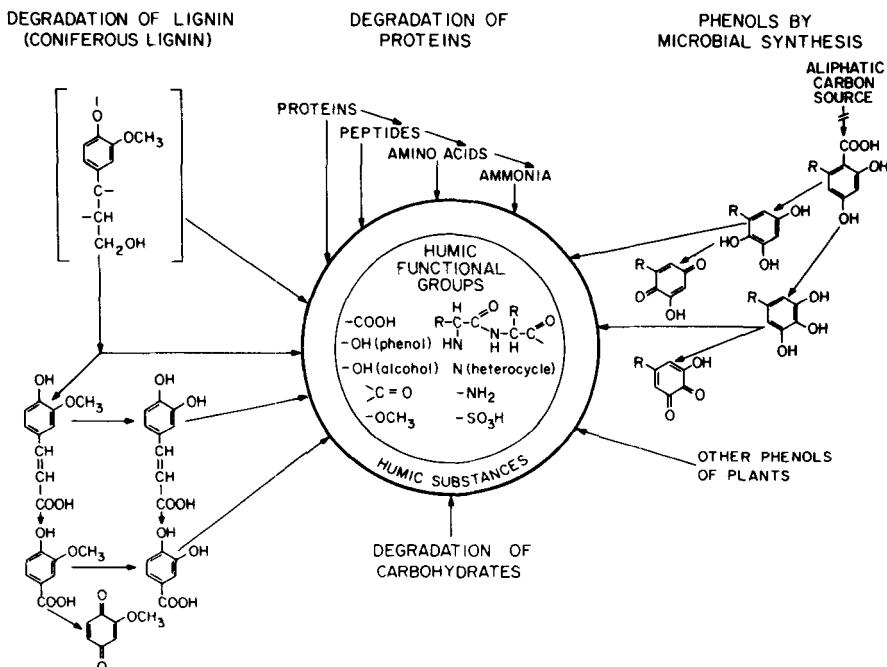
FIG. 3. Some of the phenolic compounds of soils. These hydroxylated aromatic groups could bind Si covalently, while sugars will bind through H-bonds. The existence of defined silicon compounds either in soils or in living organisms is not proven. From Sposito (1984) with permission.

Methods of study of silica structures

Given the problems of the study of amorphous materials, we must constantly look for new methods. The traditional tools are the electron microscope for an overview (Mann & Williams 1982) and infrared spectroscopy for a more detailed examination of Si–OH and O–H bonds (Iler 1979). The methods do not give clear-cut analytical results. Increasing use of solid-state NMR (Mann et al 1983) should change our knowledge of the overall composition, as described in later articles in this symposium, but we may well need to know the differences between surface layers and internal layers as well as the overall properties of the bulk silica gel.

Uptake of silicon

The movement of silicon into plants and the location of its deposition are extremely species-specific. Some plants growing in the same locality as others become heavily silicified, e.g. horsetails (*Casuarina* spp.), while others do not. The amount of water passing through the plants is similar. Thus, since it is impossible to lose silica by evaporation we must suppose that some plants exclude it or that others pump it in. Both mechanisms may operate but there is little evidence about either (Volcani 1981). How do plants control silica chemistry? Perhaps we can uncover the mechanisms by the study of diatoms and their mechanisms of uptake.

The functional value of amorphous silica

Not long ago the study of amorphous materials by chemists was considered to be almost a waste of time, since a principal preoccupation was to obtain a crystalline material so that it could be characterized analytically and by physical properties such as a melting point. An amorphous material is almost certain to by analytically variable locally and to give at best a roughly reproducible set of physical properties. It has the great advantage that it can be forced into any mould and, if it can be made to set, has no fracture planes. Biology has used many amorphous materials in its structures (Li & Volcani 1984). Examples are some celluloses (plants), chitins (insects), keratins (wools) and of course mixtures of proteins and polysaccharides (skin). The readily prepared inorganic amorphous materials are from silica and nature has made great use of them even in animals (limpets), apart from in diatoms and plants. Most animals, however, use either crystalline calcium carbonate or crystalline calcium phosphate as their biominerals and we must ask why crystallites of these chemicals were seemingly preferred to amorphous silica as life evolved. Is silica difficult to use together with proteins?

The mineral chemistry of silicon

It is not my role here to do other than introduce the mineral chemistry of silicon. At one extreme it is represented by such hard, chemically inert, materials as quartz, granites and asbestos. At the other extreme, in the clays we approach amorphous silica gels in which many –OH groups remain neither condensed to Si–O–Si bonds as in quartz nor ionized to give Si–O–M (M is a metal ion) lattices. It is important to realize that some –OH groups remain on the surfaces of all these minerals (Figs. 1 and 4). The number of –OH groups then depends on the surface-to-volume ratio, i.e. crystal size and shape. However, some forms of condensation of silica to give silicates can leave very open networks, as in the zeolites where –OH groups may be internal to the structure. Some examples of the disposition of hydroxyl groups are given in Fig. 4.

The major point about the special clay or soil minerals is their reaction with water (Mann et al 1983). It is these minerals which supply the $Si(OH)_4$ to the plants. The equilibration of soluble $Si(OH)_4$ and some of the surface of clay minerals must be quite rapid, due in part to the amorphous nature of some clay minerals and in part to the very small sizes of many particles in the clays. A very small particle has a high surface area which greatly reduces its stability and increases its solubility. Solubility is in fact a fundamental problem in silica and silicate chemistry, running through both the biological and the mineral chemistry. As we have seen, this is also confused by the hydration (hydroxylation) equilibria of the silica or silicates. Reference to a general book on clay minerals brings out this point which is so clearly applicable to silica and silicates within biological systems (Sposito 1984).

Glasses

The very common use of alkali metal silicas as glasses introduces another area of amorphous silica networks. There is an infinite variety of such materials. In general, glasses contain few –OH groups and their surfaces do not seem to have large cation-binding properties. When we compare a glass fibre with an asbestos fibre we must note the very different surface activity. Asbestos, especially in the form of blue asbestos (iron-substituted), could be a health risk not only through its physical properties but also because of its chemical reactivity. Blue asbestos is a catalyst for the H_2/O_2 reaction at high temperature but glass is not. Even glasses coloured by the incorporation of transition metals do not appear to be good catalysts, but transition metals incorporated into silica gels can be very reactive. The idea that ferruginous bodies cause damage in humans will be discussed later in this symposium.

A

1:1 LAYER

2:1 LAYER

2:1 LAYER WITH HYDROXIDE INTERLAYER

B

INNER-SPHERE SURFACE COMPLEX:
K^+ ON VERMICULITE

OUTER-SPHERE SURFACE COMPLEX:
$Ca(H_2O)_6^{2+}$ ON MONTMORILLONITE
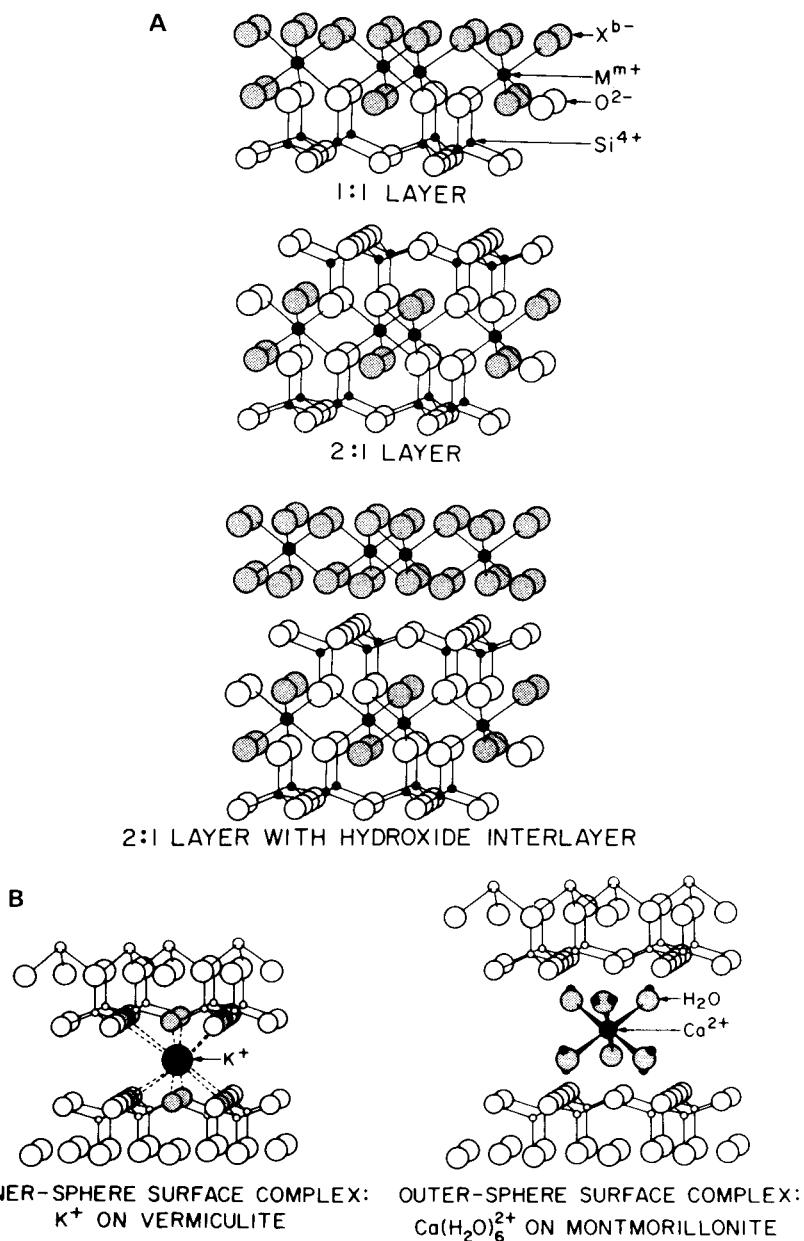
FIG. 4.(a) The surface structure of soil particles have –OH groups associated largely with high-valent metal cations except where the SiO_2 polymers terminate in irregular surfaces. (b) The disposition of metal ions in the clay minerals, vermiculite and montmorillonite. From Sposito (1984) with permission.

The irritant properties of some silicates

Animals do not appear to suffer much permanent damage from ingesting large amounts of amorphous silica. Nettle stings do not cause skin cancers but they remain a source of irritation for a day or two. It would appear that amorphous silica needles from nettles or sponges can be dissolved away. I presume that the organic protective layer of the needles is removed by enzymes and the amorphous silica then dissolves as $Si(OH)_4$. It is in this kinetic property that biological silica differs so much from the hazardous minerals, such as some aluminosilicates but not all, e.g. the clay minerals.

The hazardous minerals have been formed by long heat treatment in the earth or in industrial or experimental procedures. These minerals are not readily attacked by chemicals. Their surfaces are structures to which polysaccharides will stick. Hence once the mineral is lodged in a position in an animal system it can hardly be removed, yet it generates a response. It is this response which will interest us during this symposium. Clearly different clinical symptoms arise which may well depend on the physical properties of mineral fibres, as well as their chemistry, even down to the level of the dimensions of fibres (Volcani 1981).

Uses of silica chemistry

Most uses of silica chemistry are well known. Undoubtedly catalysts will remain a major area of exploration. However the controlled uses of silica by biology, leading to such objects as those shown in Volcani's book (1981), makes one believe that our exploitation of silica in ceramics or many composite materials is far from over. It is not a long step from the cement industry to the silica of biology. Both are largely used in building hard structural units yet they do not share delicacy of refinement at the microscopic level.

The essential nature of silica

When we use 'essential' in the context of the elements we usually mean essential to all or most species including especially humans (see Carlisle, this volume). Silicon does not *appear* to be essential for humans but probably this is due to its prevalence and the microscopic demand—essentiality is then hidden. Silica gel is not essential and is not used in higher animals. The only requirement for silica in higher animals appears to be as a cross-linking agent in some part of the connective tissue. The nature of this use is unknown but again we shall explore the problem during the discussions here. Given the major problems of connective tissue with ageing, we could ask whether the silica content of such tissue changes with age.

Conclusion

I hope I have shown in this brief introduction that the biochemistry of silicon is a very limited part of its general chemistry. Little effort has been made to exploit the chemistry of silicon, except in glasses, until relatively recently. The question arises of whether it is possible to design successful therapeutic drugs which incorporate silicon. Again this topic will be raised by others at this symposium. It is surprising how little is really known of the biological chemistry of silicon and the possibility of exploiting its chemistry in efforts to combat diseases of animals and plants.

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DISCUSSION

Last: You asked why higher organisms use calcium and lower ones use silica as structural matrix, Professor Williams. I would suggest that it is related in part to going over to a collagen-directed economy in vertebrates, providing a template to build tooth or bone or whatever. The need for calcium perhaps relates to the ambiguity of valence in silicon. There is no unique valence and if there is it is an awkward one: -1. A calcium economy however has the potential for an exceedingly specific and tight binding of calcium, which is

always going to be divalent. In cells of an organism at equilibrium with sea water it would be very awkward to have a proteinaceous template (which might be soluble), but as soon as you make an inside and outside of an organism you can go to a system where there are benefits. Does that make any sense?

Williams: Not really! I can't see how coral would be handled in such a composite scheme.

Mann: It may be dangerous to try to correlate evolutionary mechanisms with the presence of silica minerals. There is no evidence yet that silica is formed in bacteria or the monera, so how can we discuss the mechanisms at higher levels? If we wish to establish a progression of evolutionary mechanisms towards carbonate and phosphate from silica, we must first explain why bacteria do not mineralize siliceous materials.

Williams: We know that at some stage in evolution a lot of different biological minerals were present. And we know that in higher organisms such as humans and animals silica as a biomineral has almost disappeared.

Mann: That may be tied to the structural organization. As I shall show, many silica biominerals are formed in spatial compartments within cells. Such sophistication in cellular organization is not present in the monera.

Hench: At the recent workshop on Sol–Gel Science in Montpellier, France, Brinker of Sandia Labs (1985, unpublished paper) reported that a laser Raman peak has been identified that occurs uniquely in the ageing stages of silica gels, not in the sintering process. It is thought that the organization in the ageing gel of a breathing mode of silica and oxygen ions might provide an ideal site for localized mineralogical coordination of other cations. The concentration required to stabilize the surface is very low, because that stage of coordination has mostly been satisfied by the silicon. Once that mode has been created they find in the manufactured gels that the materials are then irreversibly stable to their environment. So have laser Raman studies been done on biogenic regenerated opals? If so, has anybody ever seen this unique 'breathing' mode appear?

Newman: What size was the basic particle you described, Professor Williams, and how is it formed? Would nucleation round a polysaccharide in a biogenic system be a putative mechanism? Would that have any bearing on why biogenic silica is not present in higher life forms?

Williams: In the final form in which we examined them the particles of biological origin measured from 10 nm upwards. They were bigger than the model particles of 2 nm that I described. I showed the model particle in that form as an illustration but it also presents the problem of nucleation. If one is attempting to grow a crystalline species there is a considerable barrier to nucleation. (In silica chemistry this is the barrier before getting to quartz.) The initial step has to be the putting together of a critical number of atoms in a regular repeating manner, but at first these micro-crystals have a very high surface area and are extremely unstable. They tend always to revert back into

solution, so precipitation methods for producing crystals are usually extremely slow. Amorphous precipitates, however, can be produced at very high rates from solution as soon as the solubility limits of the amorphous material have been passed. The trouble of course is that the amorphous material is much more soluble than the crystallizing material. Thus there is a thermodynamic barrier to amorphous precipitates and there is a kinetic barrier to crystallization. Biology manipulates the form of the solid laid down by manipulating the concentration, surface energy and binding kinetics. Biological systems can (1) overcome activation energy barriers to crystallization or (2) build such barriers as to obtain amorphous precipitates. Biologically evolution has selected out certain states of matter for particular purposes.

Volcani: The elemental silica particle size would theoretically be equivalent to six coordinated silicic acid tetrahedrons, and the next larger size would be six times six. Silica particle size is always a multiple of six (Greer 1971). The diatom particle seems to fit into that scheme.

Werner: I would like to add some arrows to Professor Williams' scheme (Fig. 1, p 2). One arrow would go from the lower species to silica as essential nutrient for plants and one would be for animals. And there could be arrows from lower species and from humans to silica as essential nutrient—one for pathways and one for regulation.

Volcani: In that modification it would be preferable to consider silicon as a molecular species at this stage because of its various activities. I would erase silicic acid and substitute Si-X, because we don't know what the species is. Regulation and pathways and so on are differing processes which are linked to many other processes so I would delete them from the modification.

The explanation for the absence of aluminium from this system in biology could be that aluminium is a toxic element. Organisms don't want to be killed so they tried to eliminate it in the first place.

Werner: I don't think those modifications to my modifications are very constructive. The evidence is pretty clear that silica as silicic acid is in several cell types an essential nutrient. What compound it is changed to during the uptake process is another question. If silicon is an essential element and silica an essential nutrient, there has to be a pathway. As in general biochemistry, there has to be a regulation of these pathways. I would rather stick to my scheme.

Volcani: I don't want to wipe out your concept. The problem is that each of these steps consists of processes and that scheme only concerns the element itself.

Birchall: You are saying that Si goes in as silicic acid. Nobody will dispute that, but what happens to it after that?

Farmer: Is there really a problem about concentrating silica in plants that form opal? Jones & Handreck (1967) concluded that, if grasses took up water and silica in the same proportion as in the soil solution, the water transpired and the total amount of silica in the plant balanced. But some plants grown in the

same solution kept silica out. Clovers were very low in silica whereas grasses were high. The amount of silica present in grasses did not require a biologically active pump.

Williams: Could silica ever appear in roots, according to you?

Farmer: No.

Williams: But it does appear in roots.

Mann: The idea of water movement is usually connected with transpiration, but water movement by osmoregulation across cell membranes could be a major mechanism of silicification. Water is certainly being produced in large amounts from the condensation reaction of silicic acid.

Henrotte: In vertebrates, the bones are calcified, not silicified. In fact, a reserve of calcium is necessary to maintain the stability of the blood calcium concentration, which in turn is needed for the proper functioning of the higher nervous system. If the bones were made of silicon, they could not fulfil this purpose of being a calcium exchanger. This might explain why calcium has been selected more than silicon in the course of evolution. Higher organisms need a readily available source of calcium to maintain the stability of their nervous system.

Williams: To the best of my knowledge, plant systems use calmodulins and the control mechanism for calcium in pretty well the same way as in animals. There is a parallel chemistry of calcium control that exists side by side with the silicon matrix, but plants don't make calcium phosphate.

Henrotte: But in higher vertebrates the blood calcium concentration is extremely constant. Even very small variations may impair the whole function of the nervous system.

Williams: I agree.

Carlisle: In higher animals silicon appears to have a metabolic role as well as a structural role. I think that is probably also true in most lower organisms.

Dobbie: You mentioned that aluminium is toxic, but silicon is toxic too. Later I'll show that silicic acid is very toxic when delivered to distal renal tubules and that aluminium silicates are perhaps not toxic.

Werner: The calcium requirement of diatoms is unusually high compared to that of green algae, which have a different type of cell wall (Badour 1968, Patrick 1977).

Sullivan: One reason why bacteria do not accumulate silica may be because the internal compartment of the cell is very different from the external medium, whether this is fresh water or sea water. The ions outside the cell are very different from the ions inside, owing to the influence of biological membranes at that water–cell cytoplast interface. Within the eukaryotic cell many of the siliceous structures, and possibly the higher concentrations of silicon, occur within membranes that act to sequester or isolate silicon from the rest of the biochemical machinery of the cell. It is important to keep that aspect of biology in mind. That membrane barrier is one of the many mechanisms that Professor Williams referred to for creating a special environment.

Hench: A vital aspect of collagen-apatite structures is the dramatic response to the bioelectric potential. Are there any such responses in the silica-based structures? That factor has to be taken into account in understanding the evolutionary pathways and their consequences.

Williams: The mechanism of the bioelectric potentials, the diffusion of the proton within the matrix, is certainly open to a silicate-based system. Manufactured silica systems have that possibility, therefore it was not ruled out as a possibility in evolution.

Mann: Surely there needs to be molecular anisotropy for electric polarization. You don't have such a property in amorphous silica.

Williams: If something is amorphous it can still have a distinction between its left and right side. When there is stress, as in bones, a gradient will be generated. An amorphous material is just as good from the point of view of generating bioelectric phenomena as a crystal.

Last: But the difference between the strict ionic bond in calcium phosphate and the semi-covalent bond in silicic acid would have a profound effect on electrical properties.

Volcani: In the mammalian system there is apparently a very tightly regulated homeostatic system for taking care of any increase in silicic acid in the blood. For example, in patients with silicosis the concentrations of silicon in red blood cells and serum are not increased above normal, nor is there any change during ageing. Such homeostasis is not the case in some single-cell organisms (e.g. diatoms).

Farmer: I want to comment on why aluminium is not part of life. The solubility of aluminium hydroxide is very pH-dependent; the concentration of Al^{3+} varies by a factor of 10^3 for every unit of pH. In sea water or at neutral pH its solubility is extremely low and aluminium is essentially absent from the ocean. So if life started in the ocean it had no aluminium to work on. Aluminium became freely available only as a result of plant life on the earth's surface. Its availability depends on organic acids of plant or microbial origin which decompose minerals and mobilize aluminium. Conifers growing on acid soils accumulate substantial amounts of aluminium (e.g. 500–1000 mg/kg dry matter) but they also produce phytoliths. So aluminium is tied up in some way, presumably complexed by anions such as citrate and tartarate, which form stronger complexes than does silicic acid with aluminium. Plant opal is reported to contain up to 4.5 % Al (Bartoli 1985) but in diatoms there would be none at all.

Birchall: Aluminium is not absent from the ocean. A recent paper in *Nature* (Orians & Bruland 1985) said that sea water contains at least 2 nmol kg^{-1} Al, which is not insignificant. The flux to the Atlantic ocean is 10^{-5} g Al cm^{-2} yr^{-1}.

Dobbie: I am very interested in your idea that aluminium hydroxide and silicon hydroxide are the crucial nasty substances in living organisms. Both aluminium hydroxide and silicic acid are used in pharmacy. It has long been recognized that mammals are loath to absorb these substances. Once they have

absorbed them, renal clearances are high.

Williams: The element to oxygen bond breaks to give water from aqueous species of Na^+, K^+, Mg^{2+} and Ca^{2+} but aqueous Al^{3+} and Si^{4+} can give OH^- at pH 7. The same is true for beryllium and boron, to some extent, but it is no longer true of carbon, which is moving towards the same sort of chemistry as phosphorus. Here the aqueous species break to give H^+.

Espie: Iron has a similar chemistry to aluminium, yet iron is essential.

Williams: Iron oxides in the body, however, are nasty materials, and they are locked up carefully. The amount of free iron in the body is low.

Carlisle: There is also a very efficient regulatory mechanism for iron. Under normal circumstances the body does not absorb more iron than it requires.

Sullivan: There is dissolved aluminium in sea water. The vertical distributions of silicon, germanium and aluminium in almost all sea waters appear to mimic one another. In surface sea waters that have a lot of biological activity and reduced amounts of silicon, there are also reduced amounts of aluminium and germanium. This suggests that there may be some interaction with the biological catalysts for those elements.

Werner: In relation again to the exclusion of aluminium hydroxide compared to silicic acid, we should remember that the conditions for unicellular organisms in the ocean or in fresh water are completely different from those of higher plants. Higher plants (C_4 plants) need 600 l of water to produce 1 kg dry mass. So what do we know about the exclusion mechanisms for these compounds in higher plants? Is the major exclusion mechanism in higher plants on the plasma membrane of the roots?

Williams: I agree that that is important. Obviously, as was clear from Dr Farmer's paper, we can't discuss the geological phenomena affecting silicon without discussing aluminium, because the two are interwoven in lots of different ways. But our next task is to talk about silica inside the biological system.

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Structural aspects of biogenic silica

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Abstract. The objectives of this paper are (1) to discuss the characterization of biogenic silica in terms of structural properties, and (2) to elucidate the mechanisms of structural organization within biological systems. The scale of organization is a critical factor in the characterization of biosilicification processes, and order at the nanometre, micrometre and macroscopic levels is described. Molecular order is discussed in the light of high-resolution transmission electron microscopy and solid-state NMR results obtained from samples of biogenic silica. Microscopic organization is expressed in a range of structural motifs, e.g. gels, sheets, fibres, tubes and globular assemblies, and reflects the infinitely adaptive morphology of biogenic silica. Macroscopic structures such as curved rods, spicules, perforated plates, teeth and reticular frameworks can be assembled from these microscopic motifs. The mechanisms of structural organization involve spatial (scalar and vectorial) constraints, ordered particle aggregation and chemical regulation. The possible importance of organic surfaces is discussed.

The aim of this paper is to discuss the nature of biogenic silica with reference to essential and often unique structural properties and organization. A critical factor in our approach is the characterization of siliceous structures in terms of the *scale* and *level* of organization within biological systems. Thus we assume that different processes may be involved in the generation of structural forms of nanometre (molecular), microscopic (ultrastructural) and macroscopic (bulk) dimensions. Furthermore, in order to study the properties and mechanisms of formation of biogenic silica structures we must carefully consider the interdependence of scale and biological organization.

General properties of biogenic silica

Silicon chemistry in water at neutral pH is severely limited due to the great stability of Si–O–Si units, resulting in the formation of condensed phases which may be crystalline or amorphous in structure. Since the kinetic barrier to crystallization is high (about 800 kJ mol⁻¹) amorphous phases in which the Si–O–Si bond angle can have wide variations are favoured at low temperature

and pressure. Therefore, in terms of energetics we would expect all biogenic silicas formed *de novo* to be non-crystalline and under kinetic control (it would be interesting to study biomineralization products from thermophilic organisms). The term 'amorphous silica' encompasses an almost infinite variety of structural forms, from ordered opaline aggregates to extended gel-like materials. In all these structures the mineral exists as a hydrated, covalent inorganic polymer of general formula $[SiO_{n/2}(OH)_{4-n}]_m$ (Mann et al 1983*a*). This formula, where $n=0$ to 4 and m is a large number, indicates the variation in residual functional groups within the condensed structure. There is similar variation in the extent of hydration. This flexibility in composition and reactivity indicates that biogenic silica is not a stoichiometric mineral (in the way $CaCO_3$ is, for example) and that the nature (density, hardness, solubility, viscosity) and composition of siliceous structures in biology may vary considerably, being influenced directly and indirectly by a wide variety of cellular processes.

Structural characteristics of biogenic silica

Molecular structures

Here we consider structures at the nanometre level. We are interested in the local configuration surrounding each Si atom and the possibility of local structural order within the material. We have used two techniques: high-resolution transmission electron microscopy (HRTEM), and solid-state ^{29}Si NMR spectroscopy.

(a) *HRTEM*. X-ray and electron diffraction results for so-called 'amorphous' materials show only weak diffuse diffraction maxima. Since there can be no sharp boundaries between crystalline and amorphous solids, many amorphous materials contain domains of short-range order although long-range order is absent. The extent of structural definition is thus critically dependent on the experimental techniques available. We have used HRTEM as a means of studying the structural nature of biogenic silica from plants (*Phalaris canariensis*) (Mann et al 1983b) and protozoa (*Stephanoeca diplocostata* Ellis) (Mann & Williams 1982). This technique reveals structure directly, on a local scale of angströms, and is in direct contrast to structural determination by X-ray, neutron and electron diffraction, in which the volume of sample interacting with the incident radiation is microscopically large, yielding statistical results averaged out over the area analysed.

Fig. 1 shows a high resolution 'lattice' image recorded on a siliceous costal strip in an intact lorica of the marine choanoflagellate, *Stephanoeca diplocostata* Ellis. The strip is imaged across a hole in the formvar grid, so no background noise from the carbon-coated film contributes to the image detail. Examination
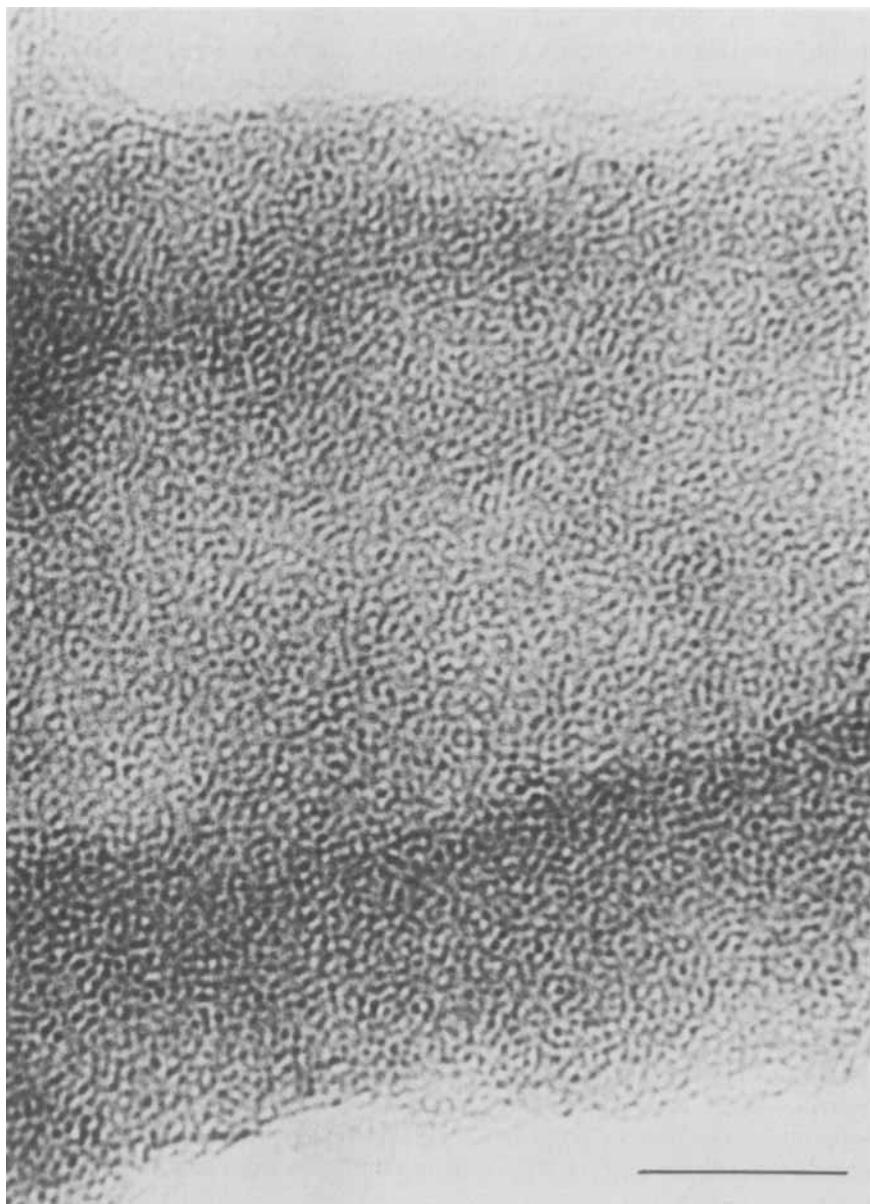
FIG. 1. High-resolution transmission electron microscope image of a costal strip in an intact lorica of *Stephanoeca diplocostata*. Bar, 10 nm.

of images such as Fig. 1 revealed irregular incoherent fringes in all areas of the siliceous biomineral. Computer simulation studies based on pattern recognition showed no conclusive evidence for regular ordering of local clusters of Si–O bonds. No short-range order could be determined extending above 1 nm (approximately three Si–O–Si units). Interpretation of fine structure below this limit is difficult, due to the thickness and focus effects on image formation. Similar results were obtained from a sample of plant silica (Mann et al 1983b). Hence the silica structures formed within these two biological systems are extremely disordered at the nanometre level.

The images indicate a structure based on a random network of SiO_4 units connected through Si–O–Si bonds of variable bond angle rather than a microcrystalline/cluster structure composed of a random array of microcrystalline polyhedra. Microcrystalline clusters would be favoured in the presence of localized crystallization centres such as extraneous cations dispersed throughout the structure. Unfortunately, we still have little information concerning the presence and possible location of trace elements in biogenic silica structures. We have used proton-induced X-ray emission (PIXE) analysis to detect trace elements in immature and mature silicified macrohairs from *P. canariensis* (Perry et al 1984a). Our results reveal that the mineralized structures contain the trace elements, S, Cl and K, distributed homogeneously throughout the mature silica fibres. Since these elements may be associated with organic phases (polysaccharides) within the macrohairs, we have at present no direct information about their molecular location or how they influence the biosilicification process.

(b) *Solid-state ^{29}Si NMR spectroscopy*. Solid state ^{29}Si NMR spectroscopy is an excellent technique for studying the local environment of Si atoms in condensed phases. The chemical shift position is highly sensitive to the coordination geometry of the Si atoms, such that, for example, the replacement of OH groups by –O– bridges can be readily detected. Since the technique is equally applicable to crystalline and amorphous materials we have used this method to study biogenic silicas isolated from plant (*P. canariensis*) macrohairs and limpet radula teeth (Perry 1985) and sponge spicules (*Halichondria panicea*).

Table 1 and Fig. 2 show chemical shifts and NMR spectra for biogenic silicas. Spectra were recorded on plant silica in the presence and absence (after acid treatment) of organic matrix components. Resonance peaks are broad in all spectra, reflecting the absence of well-defined single sites for the Si atoms, as expected for non-crystalline materials. Chemical shift results (-90 to -120 p.p.m.) confirm that only tetrahedrally coordinated Si was present (Si in octahedral sites absorbs at around -190 p.p.m.). Each sample shows a range of Si environments depending on the number of free –OH groups within the

TABLE 1   Chemical shifts (p.p.m. from Me₄ Si) for synthetic, plant (Phalaris canariensis), limpet (Patella vulgata) and sponge (Halichondria panicea) silicas

	Compound		
	$Si(OSi\equiv)_4$	$Si(OSi\equiv)_3OH$	$Si(OSi\equiv)_2(OH)_2$
Synthetic silica gel	−109.3	−99.8	−90.6
Phalaris canariensis			
Untreated	−109.8	−101.2	−92.6
Treated	−109.8	−101.1	−92.7
Patella vulgata	−113.7	−102.4	−
Halichondria panicea	−112.1	−101.8	−92.5

tetrahedral coordination sphere. Whereas the plant silica has a significant proportion of $Si(OSi\equiv)_3OH$ units, limpet and sponge silica, in contrast, are predominantly composed of $Si(OSi\equiv)_4$ units. Silylation of the plant sample with trimethylchlorosilane (Me_3SiCl), which reacts with Si–OH groups to yield Me_3Si–O–Si groupings, showed an additional resonance peak at +13.5 p.p.m. due to this moiety; however, the percentage of hydroxy groups which react (24%) indicated that not all the Si–OH groups were accessible to the silylation agent, i.e. they were not present at the sample surface (Mann et al 1983b). Thus the internal structure of the plant silica is clearly different from that for the limpet and sponge silica.

Although information is becoming available on the internal nature of biogenic silicas, little is known about the structure and properties of the surface layers. The use of techniques such as electron energy-loss spectroscopy, Auger spectroscopy and secondary ion mass spectrometry would give valuable information in this area. We have shown that the surface layers of costal strips bind Co^{2+} and Fe^{3+} when the strips are incubated with 2 mM solutions of $CoCl_2$ and $FeCl_3$ respectively (Mann & Williams 1982). Also the surface silica in these structures is stabilized with respect to demineralization, such that dissolution begins at localized centres along the central axis of the rods, resulting in tubular, brittle costal strips (see Williams, this symposium) (Mann & Williams 1982). Only at very late stages in demineralization do rods show extensive surface pitting.

Microscopic structures

Although biogenic silica exhibits no long-range crystallographic order we have recently shown that morphological order exists at the microscopic level. The initial distinction which can be made is between gel and particulate structures.
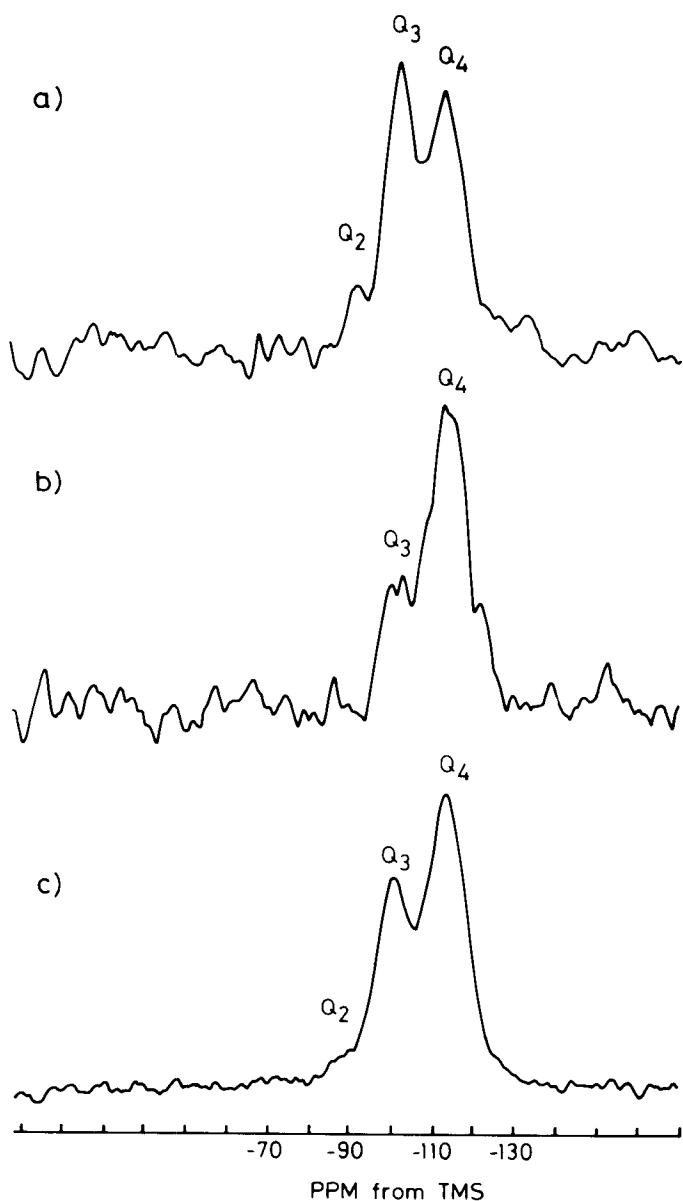
FIG. 2. ^{29}Si NMR spectra for (a) plant silica, (b) limpet silica, (c) sponge silica. $Q_4 =$ Si(OSi$\equiv$)$_4$, $Q_3 =$ Si(OSi$\equiv$)$_3$OH, $Q_2 =$ Si(OSi$\equiv$)$_2$(OH)$_2$. TMS, tetramethylsilane.

The costal strips of *S. diplocostata* Ellis are gel phases since the strip contains a continuous network of silica throughout the material. In contrast, the silica deposits in *P. canariensis* are particulate (Perry et al 1984b). Presumably many organisms can form siliceous structures containing both morphological types. The essential difference in these forms is the degree of aggregation of primary particles during the silicification process. Above pH 5 silica particles become negatively charged due to the acidity of Si–OH surface groups. Inorganic cations can bind to these surface residues, reducing the repulsive forces between particles and causing aggregation into extended gel structures. Alternatively the rate of aggregation may be influenced by the level of supersaturation, pH and the presence of organic constituents (see the section on chemical regulation, below, for details).

A surprising result in our work has been the observation that particulate forms of biogenic silica can exhibit various structural motifs at the microscopic level. In the silicified macrohairs of the grass *P. canariensis* we observed sheet-like, globular and fibrillar substructures within a single bulk structure (Fig. 3a–c) (Perry et al 1984b). Each motif comprised primary silica particles of a characteristic size, stability in the electron beam (i.e. degree of hydration) and orientation. The substructures were formed at different stages of development of the macrohairs; initially sheet-like material was deposited, followed by globular particles and finally fibrillar structures. This sequence of events appears to be linked to changes in the ionic environment (Perry et al 1984a,b) and organic matrix deposition (Perry et al 1986) within the developing macrohair.

Similar observations have been made for siliceous structures isolated from fractured limpet teeth (Mann et al 1986). Globular folded sheets and fibrillar and tubular substructures were observed, each containing arrays of primary (5–15 nm) silica particles (Fig. 3, d–f). Some structures (tubes) which contained Si showed no evidence of particulate or gel arrangements, suggesting that Si may be bound within a structural organic matrix, perhaps prior to extensive impregnation by amorphous silica. Morphological forms have also been observed in several centric diatoms (Li & Volcani 1985a,b) in which the development of the outer tube of the labiate process occurs through microfibrillar and hexagonal columnar arrangements of silicified material.

Because of the isotropic molecular nature of amorphous silica, siliceous structures have no intrinsic morphological forms but must be moulded by extrinsic factors. In this respect silica has an infinitely adaptive morphology which can be expressed in a wide range of structural motifs. The role of organic matrices or membrane substrates may be essential to the integrity of these motifs since they provide organized surfaces for silica decoration. Thus biological tissue can be impregnated and hardened without adverse effects on cell structure which could arise (in the absence of suitably designed inhibitors of crystal growth) from the growth of morphologically specific crystalline salts.
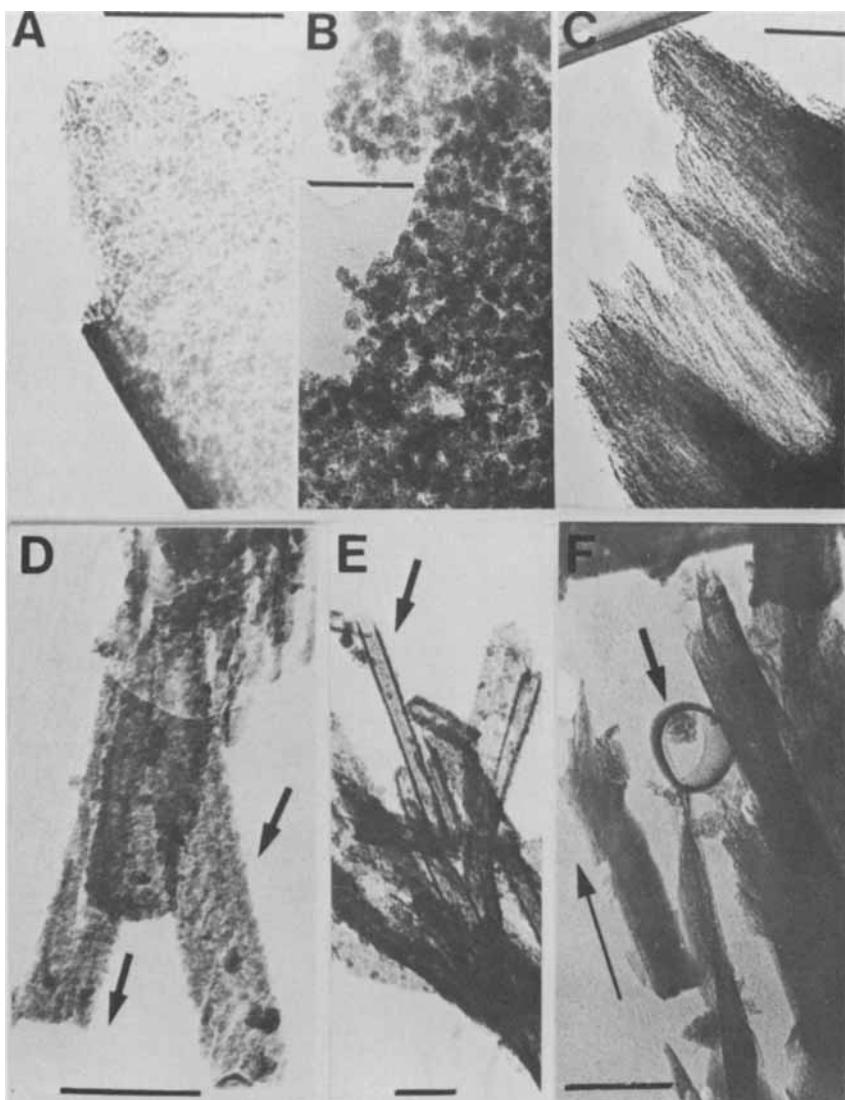
FIG. 3. Structural motifs exhibited by biogenic silicas. Plant silica: (A) sheet-like, (B) globular, (C) fibrillar (bar, 500 nm all figures). Limpet silica: (D) fibres showing helical decoration (arrows), (E) tubular structures, (F) folded sheets and tube viewed end-on (bar, 100 nm in all figures).

Macroscopic structures

Scientists have been fascinated by silicified structures since the advent of light microscopy, and the complex, often geometric, morphology.of these structures

still forms the basis for the taxonomy of a number of groups of lower plants and animals. The architectural design of these intricate structures at the macro-scopic level is mirrored in the ultrafine morphology on the microscopic scale, for example within each individual pore of the diatom frustule (Fig. 4). Clearly, control at this level of sophistication is ultimately the result of genetic processes acting on the regulation and organization of biochemical reactions at the molecular level.

The formation of diatom frustules, reticular structures in radiolaria, curved rods for lorica construction in choanoflagellates, and spicules for deterrence in sponges and plants, are all examples of the important interrelationships between mineral structure and biological function in eukaryotic cells. In contrast, no silicified structures have yet been observed in the monera. Why this should be is not clear. Since siliceous structures require the synthesis of special membrane-bounded compartments or extracellular organic matrices, the lower structural organization of prokaryotic cells may be an important
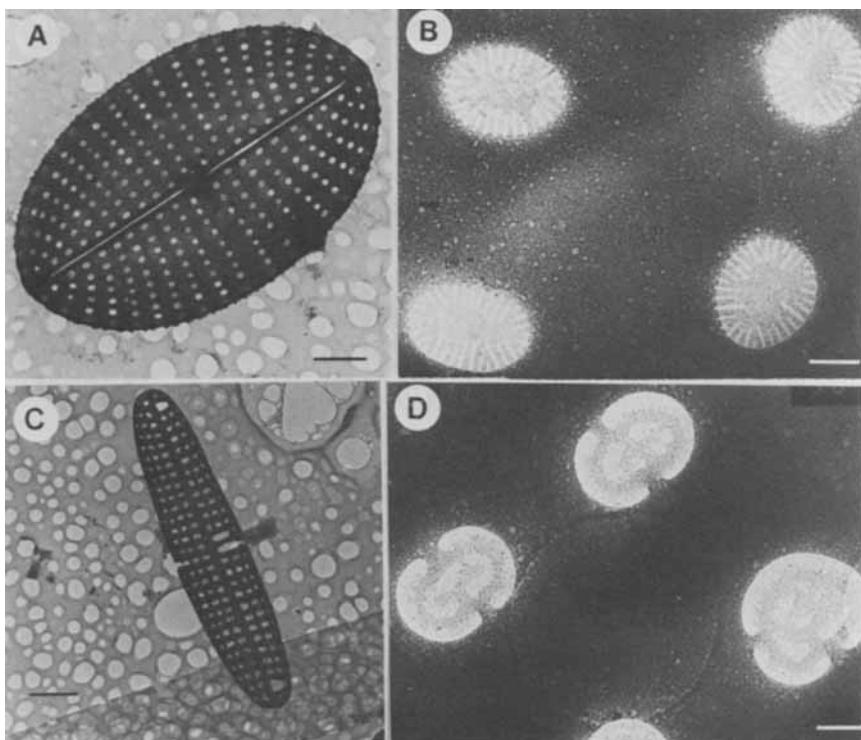
FIG. 4. Macroscopic and microscopic diatom morphologies. (A) *Cocconeis* (bar, 1.7 µm) and (B) pore structure (bar, 105 nm); (C) *Achnanthes* (bar, 2.8 µm) and (D) pore structure (bar, 110 nm).
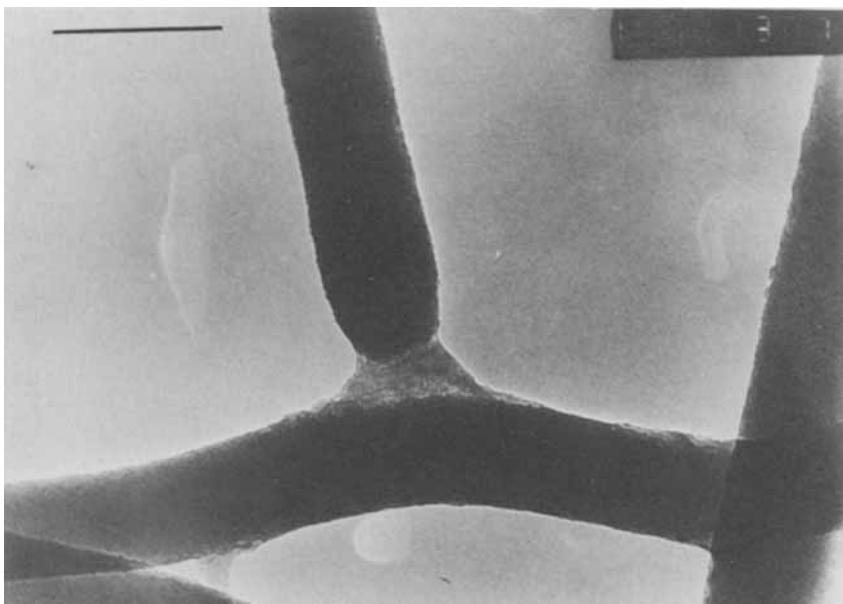
FIG. 5. T-join between costal strips in the lorica of *Stephanoeca diplocostata*. Bar, 100 nm.

factor inhibiting the formation and organization of silicified structural elements in these organisms.

Besides forming the main structural elements of many organisms, silica may also act in the linking of macroscopic structures. Fig. 5 shows the linkage found between the curved rods forming the lorica in the choanoflagellate *S. diplocostata* Ellis. The viscid material between costal strips contains silica and probably some organic material (Mann & Williams 1982). Macroscopic siliceous structures are presumably formed in biology in such a way that they function under optimum conditions with respect to the evolutionary status of the organism. An interesting implication of the preferential dissolution of costal strips in *S. diplocostata* is that the rods become hollow without significant reduction in their mechanical strength. Thus loricae remain functional, i.e. intact, even though their costal strips have undergone extensive demineralization. Only when the tubular walls become very thin will fracture and buckling occur. The mechanical design of biogenic silica is therefore an important aspect in correlating structural properties with biological function.

Mechanisms underlying structural organization

Biogenic silica, because of its intrinsic amorphous nature, can be considered as

a biomineral of infinitely adaptive morphology. The mechanisms of structural organization and the subsequent morphological forms involve spatial constraints, ordered particle aggregation and chemical regulation at the sites of mineralization.

Spatial constraints

Organic structures such as membrane-bounded vesicles are of fundamental importance in the structural organization of silica in organisms such as diatoms, choanoflagellates and radiolaria. These compartments act, in a passive sense, as spatial restraints which impart scalar and vectorial properties to the mineralization events. Thus both the total volume of silica polymerized and the direction of deposition can be precisely regulated. The construction and maintenance of stereospecific compartments in which intricate plates, curved rods and reticular frameworks can be sculptured requires complex structural processes at the cellular level, and microtubules are likely to be involved in many of these processes, as shown, for example, by the influence of microtubule poisons (colchicine) on the form of costal strips of *S. diplocostata* (Leadbeater 1984).

Ordered particle aggregation

The vectorial aggregation of preformed mineral particles has been suggested to be an important mechanism of structural organization in biominerals (Mann 1983). We have shown that in plant macrohairs and limpet teeth the macroscopic silicified material contains a range of substructural siliceous motifs (sheets, fibres and tubes) which often arise from mineral decoration of underlying organic components. In many cases the mineral takes the form of small (5–15 nm) particles which are preferentially aligned at the organic surface. The ordered aggregation of these primary units must in some way reflect the stereochemical nature, i.e. the arrangement of charged residues and hydrogen bonding centres, of the matrix surface.

Although we have no information at present on the exact stereochemistry of organic matrices involved in silicification, we have shown (Perry et al 1986) that during the silicification of plant macrohairs changes in structural organization are associated with compositional changes in the matrix components. The sheet-like silicified material deposited at the early stages of macrohair development is laid down at the same time as cellulose and heavily substituted arabinoxylan. The globular material is then laid down along with decreasing amounts of cellulose and arabinoxylan, and rapidly increasing amounts of $\beta(1-3)$, (1–4)-glucan and some mannan. The fibrillar silicified material is deposited into the mature organic phase, when further deposition of polysaccharide has virtually ceased. The differing proportions of the various polysac-

charides could provide the different chemical environments necessary to stabilize the various forms of silica observed in this system. Although it is tempting to relate these matrix compositional changes to the adoption of mineral substructural patterns, changes in the ionic environment may also play a significant role (see below).

Chemical regulation

The structural organization of biogenic silica depends on the regulation in time and space of physicochemical processes within the mineralization environment. A siliceous structure will develop from a cascade of molecular events which involve nucleation and growth (polymerization) processes. Such events are thermodynamically driven by local increases in supersaturation which can be induced by active Si transport, degradation of Si complexes, pH changes and water extrusion from enclosed volumes (reverse osmoregulation).

At pH 7, concentrations of silicic acid above 2 mM are unstable and nucleation proceeds against an energy barrier with the formation of dimers, trimers and low molecular ring structures which combine to give stable condensates of about 1 nm in diameter (Iler 1979). The presence of organic surfaces may assist nucleation, particularly if hydrogen bonding, ionic interactions or hydroxyl condensation reactions are possible between silica oligomers and the matrix interface. Covalent bonding to give Si–O–C linkages would probably require enzymic mediation at pH 7.

Although the organic matrix can play no role in epitaxial deposition, as inferred for many crystalline biominerals such as nacreous $CaCO_3$, Hecky et al (1973) have suggested a stereochemical role for protein templates rich in hydroxyl-containing side-chains (serine and threonine) in the nucleation of silica in the diatom frustule. Axial protein fibres in sponges and polysaccharide material in plant cell walls may have similar functions. Although much information is missing it seems clear that $Si(OH)_4$ has a close affinity for organic matrices which ultimately results in biogenic structures in which the inorganic and organic phases are completely integrated.

Growth of the silica phase is a process which maximizes Si–O–Si bonds at the expense of Si–OH groups. Internal condensation results in loss of water of variable degree depending on the rate and extent of polymerization. Although biogenic silica structures may vary in water content in different organisms a significant loss of water must be attained if the mineral is to have useful mechanical properties. The regulation of water fluxes is thus a major factor in silicification. In contrast, mineralization systems such as calcification do not require such precise control over water movement between biological compartments.

Growth of silica phases is determined by interfacial properties rather than crystal symmetry relationships. Thus high levels of ionic activity (0.2–0.3

M–Na$^+$ or K$^+$) within the mineralization zone will generate gel-type structures due to reduction of surface charges on primary silica nuclei. Similar effects will occur for solutions polymerized at low pH (5–7) or in the presence of hydrogen-bonding flocculants such as alcohols, proteins, lipids and polysaccharides (Iler 1978). At high pH (7–10) in the absence of cations the negatively charged silica nuclei repel each other and individual particles increase in size. Since smaller particles have a higher solubility than larger particles, growth of these silica sols occurs via Ostwald ripening processes (Iler 1979).

In conclusion, the mode of aggregation and growth of microscopic silica nuclei formed from supersaturated solutions of silicic acid can be modified by the presence of both organic and inorganic constituents within mineralization environments. Thus biogenic silicas are unique materials, showing diversity in structure, density, composition and mechanical properties (viscosity?). This is unlike crystalline biominerals, which are generally stoichiometric, have a defined density, and require interlaminated organic matrices if they are to have mechanical properties of any functional value.

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DISCUSSION

Birchall: Is there an organic template in the rods? If so, and if it is hydrogen-bonded to the silica, one would expect it to dissolve down the middle because there is less silanol condensation next to the organic matter, as the silanol groups are blocked by (probably) hydrogen bonding.

Mann: In transverse sections of the rods we often see some specific staining (two small dots in the centre of the costal strip) but it is a very small amount. I am not sure whether it is specific.

Birchall: You cannot reject the possibility of lower condensation next to any organic polymer template.

Mann: The preferential dissolution from the rod centre implies a difference in the degree of hydration within the costal strip.

Leadbeater: The problem of pattern formation is relevant to the diatoms as well. The radius of curvature of costal strips is controlled by two microtubules inside the curve but outside the silicon deposition vesicle (SDV). If you disrupt the microtubules with colchicine, the SDV is disrupted and misshapen strips are extruded. I think the shaping function of microtubules could explain the radius of curvature but what controls the diameter of the strip and the slight variation in thickness between the centre and the ends? This problem is much more considerable with the diatoms where there are many pores and areolae. Are there any biological silica systems where there is not an organic template within the SDV? Could a membrane system act purely as a mould into which silicate is pumped which then condenses within the vesicle?

Mann: Such a mechanism would suggest that precipitation occurs at the membrane surface, because of the energetics. There would be some control in the spatial sense even though there might be no specific active sites for nucleation.

Leadbeater: But from the biological point of view this would have to be controlled. Microfilaments are certainly present so they might constrict the strip and shape the pores in diatoms. I don't know if this has been demonstrated in diatoms or in choanoflagellates.

Williams: These particular systems are under a pressure from the filamentous system, which holds the shape. But holding the shape by pressure means that there is a drive expelling the water in them. The mechanism is a purely mechanical one. If the vesicle is held in this way while it is filled, then it is helping to drive the water out of the space by a mechanical device, and that changes the forces you described.

Again in a free energy diagram of a reaction of a water solution with silica, we assume the process has to be downhill, because we think in terms of crystallization in a normal layer. But could it in fact apparently go uphill? Certainly it could go uphill if we could supply energy. One way you mentioned, of pumping in materials, is against the thermodynamics of the simplest scheme but another way is to apply pressure to a certain volume. That part of space is then more likely to allow condensation than any other type of space. That is a trick that biology could have learnt.

Another way of going uphill, especially with amorphous materials, is to apply chemical energy to the material at the surface. Instead of considering silica alone we can look at the effect of a large organic surface on its stability. The silica itself would be unstable but the organic matrix plus silica is stable. The system may never reach supersaturation of pure standard silica. The surface area of these materials is large, therefore the amount of organic interaction supplies a very large amount of energy. With calcium phosphate in bone the surface area is small but here we are looking at something with a very large surface area. That would apparently drive uphill, just as pressure would.

Volcani: Dr Mann, have you any concrete indication that you really have an organic matrix in any of these systems?

Mann: We have an organic phase in the silicified plant hairs.

Volcani: Do you know anything about its composition?

Mann: It is essentially polysaccharide.

Volcani: Do you know the spatial relationship of the organic matter to the silica?

Mann: Not at the molecular level.

Volcani: The situation in the systems you described is relatively simple or primitive compared to the complexity and heterogeneity of structures in the diatoms. The matrix has interested us for some time and we have an indication that within the shell proper there is an organic structure composed of microfibrils. If we can substantiate that biochemically, all our concepts of the process of silicification in the diatom would have to be reassessed. The situation resembles to some degree that of bone matrix and its complexity. In a freeze-fractured developing cell wall of the diatom *Navicula pelliculosa* we have seen microfibril-like markings and some microtubule-like structures in the silica shell (Volcani 1981). Treating a thin section of that profile very carefully with hydrofluoric vapours dissolved the silica and left behind a residue and some microfibrils. Furthermore each diatom species has different features of the

elemental particles that constitute homologous cell wall structures. It is a very heterogeneous system which is very tightly controlled and very specialized with regard to the inorganic components. Unfortunately we cannot yet determine whether an underlying organic structure or matrix is present in this system. In the diatoms the heterogeneity of the morphological forms of deposited silica constituting the different components of the cell wall in the same organism is indeed amazing (Li & Volcani 1984).

Dobbie: Is the silica inside or outside the cell? Is it being laid down in the plants or in the diatoms? If it is in vesicles it need not be in the cells.

Volcani: The silica deposition vesicles we have seen are underneath the plasma membrane of the newly formed daughter cells and are confined to a specific site.

Dobbie: Are they connected with the surface at any time?

Volcani: That is difficult to tell from the micrographs.

Dobbie: Are there any micropinocytotic vesicles going into the vesicles?

Volcani: Vesicles are added to the silica deposition vesicles but we do not know whether they are micropinocytotic.

Dobbie: Do the vesicles appear spontaneously?

Volcani: They appear only during cell wall formation.

Dobbie: Are they formed by involution of the surface membrane?

Volcani: No; they are derived from somewhere around the Golgi apparatus but we don't know whether the Golgi is the source.

Dobbie: That doesn't tally with some of the mammalian findings. Is the vesicle preformed, with silica then coming into it?

Volcani: We did not see silica in the vesicles that fuse with the silica deposition vesicles.

Leadbeater: It is concurrent in some microorganisms but in others the vesicle is preformed and then deposition takes place.

Dobbie: Does one ever find silicon free inside the cytoplasm or does it suddenly appear in the vesicles?

Volcani: Silicon occurs in the diatom in various organelles and we found silica granules in the mitochondria not only of the diatom but of rat liver, spleen, and kidney (Mehard & Volcani 1974, 1976).

Perry: Energy dispersive X-ray analysis of sectioned macrohairs from the lemma of the grass *Phalaris canariensis* L. previously prepared after freeze substitution has shown that, at early stages of development, silicon is found in the cell vacuole (M.J. Hodson, 1985, unpublished).

Sangster: In higher plants, most of the deposits are outside the plasmalemma, except in specialized cells, or senescent cells, in which there is infilling of the lumen.

Leadbeater: The hollowing out of the costal strips to form tubes seems a little complicated. The sea water has to get into the centre of the strip initially so it has to permeate the surface of the strip. In section costal strips appear

homogeneous and they certainly are not porous. Can you comment on how the water gets in and how it gets out of the strip?

Mann: Initially we thought that perhaps the silica went down the tube, that is there was a fracture and then it dissolved down the tube. But a lot of the dissolving strips were intact. This is an interesting aspect of the design principle. If a siliceous cylinder is hollowed out a significant mechanical strain can still be maintained, even though the cylinder is still undergoing extensive demineralization.

I imagine that water percolates through the gel structure but again that leads to all sorts of conceptual problems about what is stabilizing the surface and how water gets through and comes out. So my answer is that I don't know.

Leadbeater: It is disconcerting that something that looks homogeneous in section can be so permeable.

Werner: There are a number of questions about animal cells compared to plant cells. What was pointed out here for silica condensation on the organic matrix in plants is closely connected to the biosynthesis of plant cell walls in general. It is a general scheme that the Golgi is involved in the biogenesis of plant cell walls. But even here we still don't even know how many glucan synthases are involved in cellulose synthesis. A large number of other polysaccharides also participate in cell wall synthesis in plants. How much of this is replaced by a silica matrix apparently depends on the species. It can be up to 95% in some diatom species, and even in diatoms there are cases where less than 5% of the cell wall is silica and 95% is organic material. There is almost a continuum of low concentrations of silica in cell wall biosynthesis up to very high concentrations. I think the mechanism is always pretty much the same and in this context we have to admit our incomplete knowledge of cell wall biosynthesis. This emphasizes again that silicon mineralization should not be separated from progress in our knowledge of the biochemistry of cell walls.

Birchall: The rods dissolving down the middle remind me of what happens in a glass fibre polymer composite. In an apparently intact interface one can't see any gap at all, but water goes right down the glass–polymer interface and debonding is rapid.

Mann: Does it go down the centre?

Birchall: No. This is a glass fibre sitting next to a polymer in a composite. It looks absolutely solid. I am tempted to say there must an organic molecule in the rods you show and a low silanol condensation at the interface. This is attacked by water so that the rod dissolves down the centre.

Williams: How much carbon is there, analytically, in what you call the silica matrix?

Volcani: The complication of the diatom is that the mature cell wall consists of two components: an organic component is contiguous topologically and otherwise to the mineral component. The chemistry of the organic component is extremely complex. It is predominantly made of proteinaceous and polysac-

charide material, we know how much of this material is there, and we know that it differs from species to species. We know the composition of the constituents (amino acids, sugars, lipids) and so on, but we also know that starting from the birth of the wall there is a constantly varying composition in the synthesis of that final product which I called the organic casing. Because of this, that unit membrane, the silicalemma which constitutes the silica deposition vesicles, is constantly going through changes. The chemistry of the casing differs so much from what the silicalemma started with, and we don't know whether the silicalemma serves as the silica matrix, nor the processes involved in silica deposition *per se*. The chemical composition unfortunately cannot be related to any single phase in the course of wall development.

Willams: So in the electron microscope at the highest resolution, or by any method of staining, you have not seen whether these thin fibres go all the way through the silica matrix?

Volcani: What we do see, depending on the species, is that there are variations in the morphology of the siliceous substructures, i.e. they are hairy, or spherical, or fibrillar and so on, but this doesn't tell us whether there is any organic material there, nor does it tell us the properties, chemical or otherwise, of that membrane within which silicification takes place from the beginning to the end.

Richards: Have you used chemical or other means to try to alter the high resolution picture you showed?

Mann: We can try to do pattern recognition. We could also take Fourier transforms and optical bench experiments, though we haven't done that yet. We have corresponding electron diffraction patterns of the material, which again clearly indicate that there is no order on the nanometre scale. The problem with that system is that the rods are rather thick. We really need to look at material about 1 or 2 nm thick during this processing. I could do image processing and get clearer pictures but whether they would correspond to the atomic potential of the sample is another question altogether.

Perry: Pattern recognition work has been done on micrographs of plant silica by computer simulation. At the nanometre level no ordered patterning was observed. The same technique was used with micrographs of the different substructural arrangements of particulate silica. This work confirmed that the degree of orientation decreased as the silica passed from fibrillar to sheet-like to the globular arrangement.

Hench: Are the amorphous hydrated biological silica structures stable to crystallization, with the high pumping capacity of the TEM vacuum and the high heating effects of the TEM beam? In glassy silicates we have seen beam heating effects on crystallization kinetics that are approximately equivalent to 80–100°C (Kinser & Hench 1970). This would imply that these biological silica structures are unusually stable under the high vacuum TEM conditions, and there is some very big kinetic barrier for their crystallization and dehydration.

Perry: The different particulate arrangements of silica do vary in stability as determined in the electron beam. This may be correlated with the degree of hydration of the different structural morphologies.

Hench: That could be a real clue to understanding the ultrastructural organization of biological silica.

Mann: The mechanism of transformation to a crystalline state is likely to be a dissolution re-nucleation phenomenon, so the environmental conditions for transformation are important.

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Silicification by diatoms

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Abstract. The species-specific, genetically determined silica frustule of diatoms provides an excellent eukaryotic model for understanding the process of biomineralization and how it is regulated. An effort is made to define molecular events and biochemical constituents in silicon mineralization and to correlate these events with those aspects of silica frustule morphogenesis which have been reported in the published work. A sequential series of steps of silicic acid interaction with the cell is proposed which might be expected for silicon metabolism leading to cell wall formation. A model is put forward that provides for a conceptual framework for continued studies in this area, and provides testable hypotheses which should lead to a greater understanding of biomineralization processes in general and silicification in particular.

The long-range goal of the studies reported here is to construct a theoretical and experimental model of biomineralization through investigation of silicification in diatoms. An attempt has been made to synthesize information about possible molecular events and biochemical constituents involved in silicon mineralization. These events are correlated with those aspects of silica frustule morphogenesis which are reported in the published work. Such studies may also shed some light on basic questions of diatom symmetry and frustule architecture as well as on aspects of diatom evolution.

The model of silicification developed here postulates a sequential series of steps of soluble silicon (silicic acid, $Si(OH)_4$ or silicate, $SiO(OH)_3^-$) interaction with the cell. These steps are listed in the possible order which might be expected for silicon metabolism leading to cell wall formation.

(1) Exocellular binding of Si to a cell wall, 'periplasmic' protein or plasmalemma site (Blank & Sullivan 1979).
(2) Transmembrane transport of Si at the plasmalemma by an active or group translocation mechanism which is promoted by Na^+ or K^+ or both (Azam et al 1974, Sullivan 1976, Bhattacharyya & Volcani 1980, 1983).
(3) Formation of soluble Si pool(s) and its (their) regulation (Azam et al 1974, Sullivan 1976, 1977, 1979, 1980).

(4)   Chemical modification of the transported Si species to partially poly-merized forms (dimers, trimers, etc.) (Volcani 1978, Sullivan 1980) or to organosilicon compounds (Bhattacharyya & Volcani 1983).

(5)   Packaging or sequestration of Si compounds into subcellular organelles such as mitochondria, chloroplasts or vesicles derived from the Golgi or endoplasmic reticulum (Mehard et al 1974, Schmid & Schulz 1979, Schmid 1985).

(6)   Microtubule-directed translocation of Si sequestered in membrane-limited vesicles (silicon transport vesicles), to the site of wall formation (Schmid 1980, Pickett-Heaps et al 1979, Blank & Sullivan 1980).

(7)   Direct transport of Si into the silicalemma (Volcani 1981) or the coalesc-ence of membraneous 'packages' of silica-laden vesicles which collectively form the silicalemma (Schmid & Schulz 1979, Schmid 1985).

(8)   Nucleation and epitaxial growth of Si monomers on a template within the silicalemma (Hecky et al 1973, Pickett-Heaps et al 1979) or complex polymerization within vesicles, leading to Si macromolecules of $> 10^6$ daltons which may be the building blocks of the diatom frustule, and colloidal assembly of these to form the silicalemma containing the growing frustule (Greer 1971, Sullivan 1980).

(9)   Orientation and positioning by microtubules/microfilaments of the sili-calemma and/or orientation by microtubules of the silica deposition vesicles which coalesce to form the silicalemma and frustule (Schmid 1980, Blank & Sullivan 1980, 1983). Shape determined by vesicle moulding of silicalemma (Schmid 1984, 1985) and/or orientation and positioning by microtubules.

(10)   Termination of polymerization and/or colloidal assembly which results in the complex architecture of the silica valve followed by encasing of valve in an organic matrix external to the silicalemma. Completion of silica valve proper (Volcani 1981).

(11)   Extrusion of valve through plasmalemma (Chiappino & Volcani 1977, Crawford 1981).

(12)   Girdle band and intercalary band formation, extrusion through plas-malemma and attachment to the encased silica valve (Chiappino & Volcani 1977) to complete frustule formation.

(13)   Cell wall completion and daughter cell separation.

Experimental evidence is presented to substantiate some of the early events, regulatory aspects, energetic and macromolecular requirements described in the silicon biomineralization model. Several excellent reviews of silicification by diatoms have emphasized ultrastructural aspects of morphogenesis (Volcani 1981, Schmid et al 1981). I shall therefore focus on biochemical aspects of silicification, in keeping with the theme of this symposium.

During a brief but discrete period of their cell cycle, diatoms mineralize a

morphologically complex, species-specific silica shell (valve) as the major structural component of their cell wall. This rigid valve is composed of hydrated, amorphous silica $(SiO_2 \times H_2O)_n$, referred to as biogenic opal, and it may constitute from a few per cent to as much as 50% of the dry weight of various species (Lewin & Guillard 1963).

How do diatoms make these architecturally elegant silica valves? Obviously, from the above description, valve morphogenesis is a highly regulated process. The species-specific nature of the valve shows clearly that it is genetically determined rather than being organized strictly by molecular or ionic forces, as would happen in crystalline structures. Note that the biogenic opal of diatom valves is, in a chemical sense, amorphous and does not show a well defined X-ray diffraction pattern (Kamatani 1971). What I am emphasizing here is that

$(SiO_2)_n$

$k_4?$ k_3

Silicon n – 1

Silicon 4

Silicon 3

Silicon 2

$Si(OH)_4$ 1

k_1 k_2

$Si(OH)_4$

Biogenic opal of silica valve
~46 fmol Si cell^{-1}

Soluble cytoplasmic pool(s)
of silicic acid and derivatives
~6 fmol Si cell^{-1}

Environmental silicic acid
1 – 500 μmol l^{-1}

k_1, unidirectional influx of $Si(OH)_4$ into cell;
k_2, unidirectional efflux of $Si(OH)_4$ out of cell;
k_1–k_2, net flux of $Si(OH)_4$ into intracellular pool (transport);
k_3, silicon in flux to valve (incorporation);
k_4, silicon release (dissolution) from valve;
k_3–k_4, net silicon incorporation (growth of valve).
(fmole = 10^{-15} mol)

FIG. 1. Schematic representation of proposed sequential chemical transformations during silicic acid metabolism in *Navicula saprophilia*. See text for description.

the mineralogical nature of the silica valve is non-crystalline; thus its chemical nature is not likely to contribute inherently to the overall structure and pattern of the valve at the morphological level by which diatom species are defined. Yet at a smaller scale, that of monomers, macromolecules and colloidal particles, the chemistry of silicon undoubtedly plays an important role in the structure of the basic units from which the valve is ultimately assembled.

In a very brief and highly simplified view, diatoms actively 'capture', by binding, soluble Si from their external aqueous environment, where the ambient concentrations are presumably too low to allow diffusion to fulfil their enormous needs for the element. For instance, sea water $Si(OH)_4$ concentrations are about 1–100 μmol l^{-1} and freshwater concentrations are about 10–500 μmol l^{-1}. After binding to some site on the external surface of the cell, Si is actively transported across the plasma membrane to the cytoplasm, where it forms a pool of Si. Once inside the cell it may, through a variety of chemical/ biochemical reactions, be converted to Si polymers and/or organosilicon compounds and these may be sequestered in various subcellular compartments which are likely to be membrane-bounded. An unknown number of metabolic steps leads to the final polycondensed form of Si which is the silica, $(SiO_2 \times H_2O)_n$, valve (Fig. 1). I proposed a similar model for silicification at the 5th International Symposium of Living and Fossil Diatoms (Antwerp, 1978, unpublished) and an updated version is shown in the introduction to this paper.

In the following pages is presented a review of selected papers from the published work which present evidence for the early steps of silicification by diatoms.

Exocellular binding of $Si(OH)_4$ to a cell wall, 'periplasmic' protein or a plasmalemma site

Background

The initial interactions of inorganic ions or small organic metabolites with a cell often involve a binding to site-specific molecules on or near the outer surface of the cell. This binding and the transmembrane transport that follows it are the first two steps in the metabolism of a solute.

It was pointed out by Kalckar (1971) that 'substrate recognition by specific binding of ligands to be transported must be the first event in active transport as well as in facilitated flow and counterflow as catalyzed by permeases.' Techniques for the release of bacterial membrane proteins have led investigators to isolate binding-protein activities for sulphate, glutamine and galactose. Binding activities of iron and copper have also been demonstrated for bovine heart mitochondria and calcium binding by rat mitochondria.

In vivo binding studies

Evidence for *in vivo* Si binding by diatoms was obtained by Blank & Sullivan (1979) when whole cells of *Nitzschia angularis* were exposed to label, ^{68}Ge-Si(OH)$_4$, for 3 s. After hyptonic cell lysis, the water-soluble and non-soluble cell fractions were separated by high speed centrifugation (50 000 g h^{-1}). The results suggest that the binding sites may be on cellular membranes, since *in vitro* binding did not occur to cell wall fractions and since the 50 000 g pellet contained both cell walls and membranes. Binding to a membrane site was confirmed in studies with a congener of *N. angularis*, *Nitzschia alba*, to be mentioned later.

Blank & Sullivan (1979) also estimated the number of binding sites present in *N. angularis*, assuming that net radiolabel present (after 3-s uptake) in the pellet of lysed cells is bound at specific sites and that Si binding occurs on a 1:1 molar basis at those sites. An estimate of binding sites yielded 1738 ± 133 binding sites /μm^2 or 1.4 × 10^6 binding sites per cell. The calculation was made using a surface area of 800 μm^2 cell^{-1} and specific activity of 110 d.p.m. ^{68}Ge/pmol Si(OH)$_4^{-1}$. This seems to be a reasonable number of sites, since others have reported 6000 Na$^+$/K$^+$–transporting ATPase sites/μm^2 in rabbit outer medulla membrane and 7.5 × 10^5 ouabain-sensitive cation transport sites for HeLa cells. Large numbers of binding sites might be expected for Na$^+$ and K$^+$, from the roles and relative abundance of these important cations in cellular metabolism and osmoregulation. Sullivan & Volcani (1974) have reported the presence of an Na$^+$/K$^+$-ATPase in plasmalemma of *N. alba*, a congener of *N. angularis*. Recent work in Volcani's laboratory has further implicated Na$^+$/K$^+$-ATPase in silicon transport (see page 66). Diatoms, which may contain as much as 50% SiO$_2$ on a dry weight basis, require a massive influx of Si during a discrete period of their division cycle for building the silica frustule (Sullivan 1977). We therefore calculated the turnover time of Si on the binding sites of *N. angularis* by two methods. The first assumed 2 × 10^{-13} moles Si per cell wall and a 2–h period of frustule formation, from the physiological data of Sadava & Volcani (1977); these assumptions give a value of 12 molecules Si(OH)$_4$ bound/s. Using the V_{max} reported by Blank & Sullivan (1979) of 560 pmol 10^6 cells^{-1} min^{-1} and 1.4 × 10^6 binding sites per cell we estimated that four molecules of Si(OH)$_4$ were bound/s, which is in reasonable agreement with the physiological findings noted above.

In vitro binding studies

Using the isolated membranes from protoplasts of *N. alba*, I was able to show selective binding of ^{68}Ge(OH)$_4$ to diatom membranes by the technique of equilibrium dialysis (Table 1) (unpublished). Among the membrane fractions

TABLE 1   Equilibrium dialysis of membrane fractions[a] isolated from Nitzschia alba with ^{68}Ge(OH)$_4$

Membrane fraction	Enriched in	Specific activity (d.p.m. ^{68}Ge(OH)$_4$/ mg protein)
I	Endoplasmic reticulum	26 797
II	Golgi	11 554
III	Unidentified	5 457
IV	Plasmalemma	21 101
V	Unidentified	11 022
P–2	Mitochondria	2 181
Control	Fraction V Sigma BSA[b]	303

[a] Membrane fractions isolated according to Sullivan & Volcani (1974).
[b] BSA, bovine serum albumin.

tested, the fraction enriched in plasmalemma (Fraction IV) and endoplasmic reticulum (Fraction I) showed the highest specific activities (d.p.m. ^{68}Ge(OH)$_4$/mg membrane protein) of label associated with them. Such an association or binding might be expected for a component of the Si transport system such as the silicon transport vesicles, silicon deposition vesicles, plasmalemma (Fraction IV), or silicalemma. Fraction I enriched in endoplasmic reticulum is postulated to be the origin of the proposed silica vesicles which fuse to form the silicalemma containing the growing silica frustule (Chiappino & Volcani 1977, Schmid & Schulz 1979).

Transmembrane transport of Si at the plasmalemma

Background

As early as 1954 Dr Joyce C. Lewin demonstrated that the freshwater diatom, *Navicula pelliculosa* (recently revised to *Navicula saprophilia*), rapidly takes up Si from its growth medium, and she suggested that thiol groups in the cell membrane may be involved in the Si uptake process. In later reports she showed that monomeric silicate or silicic acid, but not the organo–silicon compounds (Lewin 1955a) she tested, are used as sources of Si and that uptake of Si is linked to aerobic respiration (Lewin 1955b). In these studies, cells were incubated for periods of hours and loss of molybdic acid-reactive silicic acid from the medium was determined colorimetrically. These results can, therefore, be interpreted only in terms of total Si metabolism, not of initial transport of Si.

Characterization of in vivo transport using silicon and germanium–silicon

Silicic acid transport *per se* was clearly demonstrated in *N. alba* by Azam et al (1974), using $^{31}Si(OH)_4$. They characterized silicic acid transport in this heterotrophic diatom as a carrier-mediated membrane transport system with Michaelis–Menten type saturation kinetics and a Q_{10} of 2. By studying initial rates of uptake, rather than steady-state rates of metabolism, they were able to demonstrate that metabolic energy was required for the net uptake of Si and this was taken to suggest that an active transport system may be involved.

In a follow-up study Azam & Volcani (1974) examined $^{68}Ge(OH)_4$ transport in *N. alba* under Si-free conditions in an effort to temporally separate the transport step from subsequent metabolism of the chemical species. This can be accomplished, since $^{68}Ge(OH)_4$ *in the absence* of $Si(OH)_4$ is not incorporated into acid-insoluble material and therefore presumably is not further metabolized. (Note different behaviour of $^{68}Ge(OH)_4$ in the presence of $Si(OH)_4$, next paragraph.) They concluded that $Ge(OH)_4$ is transported actively at the expense of metabolic energy by two transport systems: a high affinity–low capacity and a low affinity–high capacity system; Si may be similarly transported. In a later report Azam (1974) presented findings which 'are consistent with the interpretation that $^{68}Ge(OH)_4$, when present in trace amounts relative to $Si(OH)_4$, acts as a tracer of $Si(OH)_4$ uptake and assimilation in the diatoms *N. pelliculosa* and *N. alba*.' This paper (Azam 1974) forms the foundation for the validity of the $^{68}Ge-Si(OH)_4$ technique. The technique is supported by several subsequent reports (Sullivan 1976, 1977, 1979, 1980) and by unpublished studies from my laboratory by Dr A. Palmisano, who examined the kinetics of silica valve dissolution by release of molybdate-reactive Si and ^{68}Ge from cells uniformly labelled for 10 generations with $^{68}Ge-Si(OH)_4$.

Using the $^{68}Ge-Si(OH)_4$ technique, I characterized the Si transport system and its regulation during growth in batch culture of the photosynthetic diatom of *Nav. pelliculosa* (Sullivan 1976). These findings are compatible with the idea that transmembrane transport of Si is a carrier-mediated, energy-dependent process, but whether the mechanism could be described as active transport or a group translocation could not be definitively stated. Recent studies in my laboratory have, however, given strength to the interpretation that this is an active transport process, since at least 50% of the Si pool appears to be unmodified silicic acid, which is rapidly effluxed from the cell in the presence of 2,4-DNP (Sullivan 1980).

Another interesting and provocative feature of the Si transport in *Nav. pelliculosa* is its dependence on Na^+ and K^+ in the uptake medium, which correlated well with its inhibition by valinomycin, a cyclic dipeptide known to dissipate K^+ gradients. These findings, coupled with the demonstration of an Na^+/K^+-ATPase in *N. alba* plasma membrane (Sullivan & Volcani 1974), the lack of $Si(OH)_4$ uptake in K^+-free medium by *Cyctotella cryptica* (Roth &

Werner 1978), and evidence of Na^+-coupled transport of amino acids by Hellebust (1978), suggested a possible mechanism for Si uptake at the molecular level. Further studies were done by Bhattacharyya & Volcani (1980, 1983) using an *in vivo* and *in vitro* system derived from *N. alba*.

Silicon transport model involving Na^+ symport and ionophores

Bhattacharyya & Volcani (1980) demonstrated an Na^+-dependent uptake of ^{31}Si by silicon-starved *N. alba* cells (a marine diatom). No other ions, including K^+, Rb^+, NH_4^+, Li^+ and choline, stimulated uptake, indicating a specific Na^+ requirement for Si transport. Bhattacharyya & Volcani (1980) also reported ^{31}Si uptake from an Na^+/K^+-ATPase rich fraction of membrane vesicles prepared from *N. alba* cells (Sullivan & Volcani 1974). In the isolated vesicles, ^{31}Si was taken up only when an Na^+ gradient was imposed across the membrane or when it was generated by cytoplasmic Na^+/K^+-ATPase. Na^+ gradient-dependent uptake was inhibited by monensin but not by carbonyl cyanide-*m*-chlorophenyl hydrazone, valinomycin or vanadate. Uptake increased if an internally negative potential was imposed across the membrane, which suggests that Si transport is electrogenic. Bhattacharyya & Volcani (1980) explained their results by a model adapted from Crane's Na^+-gradient hypothesis (Crane 1977) in which silicate is symported with Na^+ and the transport system is driven by the Na^+ gradient that is generated and maintained across the membrane by the activity of Na^+/K^+-ATPase. This important study was followed by one in which Bhattacharyya & Volcani (1983) isolated silicate ionophores by high-pressure liquid chromatography (HPLC) from *N. alba*. The ionophores induced $^{68}Ge(OH)_4$ across a bulk-organic phase and $^{31}Si(OH)_4$ uptake by synthetic lipid vesicles. The activity depended on the presence of Na^+ in the assay mixture.

Unfortunately only a preliminary chemical characterization of the ionophore, which yielded little information, has been reported. While Bhattacharyya & Volcani (1983) did not claim to fully understand the biological significance of the ionophores in *N. alba*, they speculated that one or both ionophores could be involved in the active membrane transport of silicate, as cytoplasmic silicate carriers or as sequestering agents to retain and regulate the Si pool. Future studies should be directed at determination of the structure of these novel molecules, since along with β-thujaplicine they are one of the few natural organic compounds thought to complex specifically with silicon.

Regulation of silicon transport and the cell cycle

The regulation and timing of Si metabolism during the diatom cycle is a most intriguing problem. It is also an important demonstration of the highly regulated fashion by which diatoms exercise biochemical, metabolic, mor-

phogenetic and temporal control of biomineralization processes during their division cycle. As part of an effort to understand how Si mineralization is regulated in diatoms, I investigated changes in Si transport rates during the 12-h cell cycle of synchronized populations of *Nav. pelliculosa* (Sullivan 1977). This study revealed a 10-fold increase followed by a decrease in transport rate during the cell division cycle; the transport rates were therefore shown to be highly dependent on cell cycle stage and predictably revealed this 'peak' pattern.

Two major questions arose as a result of the observed 'peak' pattern of transport rate.

(1) By what cell regulatory process (or processes) is this pattern determined, i.e. how is the dramatic 10-fold increase and decrease in transport rate brought about?

(2) What triggers the transport rate change in such a way as to confer temporal regulation, i.e. how is the 10-fold increase and decrease in transport rate brought about at specific times during the cell division cycle?

The experiments reported went further towards answering the first question than the second. The rise in transport rate (hours 2–5) required *de novo* protein synthesis and the fall in rate (hours 6–9) might be caused by inhibition of specific protein synthesis. The postulated protein component of the transport system is believed to be metabolically unstable, with a half-life of 1–2.2 h. The rise in transport activity did not appear to be regulated by an induction or derepression-type mechanism, since the rate increased in the presence or absence of Si in the medium. However, kinetic analysis showed that there were significant changes in K_s values, indicating an increased 'affinity' for Si as cells neared maximal uptake rates. The changes in transport rate were also reflected by increases in V_{max} values which are interpreted as an increase in the numbers of functional transport (and/or binding) 'sites' in the plasmalemma. However, proof of this interpretation awaits isolation of the transport system and quantitative studies of changes in its concentration within the plasmalemma.

Thus a dual mechanism of regulatory control appears to be operative: (1) the transport system (or a component of it) appears to be preferentially synthesized at cell cycle times (hours 3–6) when a large influx of $Si(OH)_4$ is required for silica frustule formation. As the transport system is not replaced, it is then allowed to 'decay', as a result of its instability, once its function of supplying Si for frustule building has been filled hours (hours 6–9). (2) The nearly synthesized transport system or an already existing system (or both) appears to be modified in terms of increasing its 'affinity' (K_s) for $Si(OH)_4$ at hours 3–5. It is not known what specific factor(s) control these regulatory processes; yet, largely because the transport rate depends on the cell cycle stage, the findings appear to fit the 'dependent pathway' model proposed by Hartwell et al (1974) to explain oscillations in enzyme synthesis during the cell cycle. In the present case the Si transport system is considered analogous to an enzyme. Mitchison

(1974) has pointed out that there are several possible relationships between four observable events in a temporal sequence through a cell cycle. When successive events cannot occur unless the preceding event has occurred the sequence is referred to as a 'dependent sequence' and is essentially analogous to what Hartwell calls a dependent pathway. This terminology may be more rationally applied to both biochemical events and morphological events such as mitosis or, in this present case, valve morphogenesis. The available evidence suggests that the major events in the division cycle of *Nav. pelliculosa*—DNA synthesis, mitosis, nuclear division, cytokinesis, and silicification (i.e. silica valve and cell wall morphogenesis)—lie in a dependent pathway. The reader will see, however, from the work of Schmid (1980), Blank & Sullivan (1980) and Pickett-Heaps et al (1979) that this is a simplistic view and that events, probably involving microtubules, intimately yet subtly connect mitosis with silicification.

The nature and size of silicon pool(s)

While not directly addressing the question of conventional concepts of soluble Si pools. Werner (1966) attempted a partial fractionation of the Si pool of *Cyclotella cryptica* grown under conditions of Si or light deficiency. Werner distinguished three silicic acid fractions: (1) total silicic acid (TSA); (2) plasmatic silicic acid (PSA); (3) acid-polymerizable silicic acid (APSA). These fractions are distinguished by various (non-sequential) methods using whole or lysed cells of *C. cryptica*. TSA was determined by treatment of whole cells with 0.2 M-NaOH for 15 min at 100 °C, neutralization with H_2SO_4 and colorimetric determination of silicic acid.

Werner determined the amount of silicic acid in a fraction solubilized in H_2O or 0.1 M-HCl (both 15 min at 100 °C) from a three times precipitated (with 66% saturated $(NH_4)_2SO_4$) cytoplasmic supernatant fraction (5000 g, 8 min) of sonically disrupted cells. This was the material referred to as PSA and its solubility properties were shown to differ from cell wall silica; Werner suggested that the PSA was protein-bound Si and was localized in the cytoplasm or in cell organelles. Note that if there had been any free silicic acid or Si compounds in the cytoplasm that were not precipitable by $(NH_4)_2SO_4$ they would have been discarded in supernatant III (see Scheme I, Werner 1966). It is important also to note that the PSA of silicic acid-deficient cells decreased by 50–90% during Si starvation.

Werner also examined silicic acid extracted from whole cells by hot H_2O extraction and by 0.1 M-HCl at 100 °C; after measuring the difference between these two, he suggested the presence of a third silicic acid fraction (APSA) which disappeared from cells incubated for 2 h in silicic acid-free medium in the light. Unfortunately the methods Werner used do not allow a direct way of rigorously distinguishing between these three fractions, since he used different

batches of cells for each fraction rather than a sequential extraction of a single batch. Thus, since PSA and APSA are operationally defined on different batches of cells, they may be the same pools, or at least a portion of PSA may be included in APSA and *vice versa*. Also, the name given to APSA (acid-polymerizable silicic acid) may be inaccurate: silicic acid was not demonstrated to polymerize; it was assumed to polymerize inside the cell, since it was not detected in extracts by reaction with molybdic acid. This point is made since acid treatment might be expected to precipitate a protein or polysaccharide and its associated Si, or it might render Si inside a membrane vesicle non-extractable. These possible interpretations of the findings should also be considered.

Coombs & Volcani (1968) demonstrated that various amounts of [31]Si could be extracted from intact *Nav. pelliculosa* with hot or cold solvents. The efficiency of [31]Si extraction by various solvents 1 h after $Si(OH)_4$ uptake was: hot ethanol > hot trichloroacetic acid > cold trichloroacetic acid. Coombs and Volcani postulated, as Lewin (1955a) and Werner (1966) had previously, that silicon was actively accumulated as Si–organic compounds. They interpreted the labelled silicon extracted in their experiments as being [31]Si 'leached from the newly developing walls or that derived from metabolic pools of Si-containing compounds.' Their attempts to characterize putative Si–organic compounds were unsuccessful.

In more detailed studies of [31]$Si(OH)_4$ transport in *N. alba*, Azam et al (1974) determined the presence of a rapidly equilibrating (1–2 min) extractable Si pool. They showed that after a 10-min uptake of labelled silicic acid followed by hypotonic cell lysis, 90% of the label is in a cell-wall-enriched fraction ($3000\,g$, 3 min) and 5.2% is in the 100 000 g 30 min soluble supernatant fraction. About 80% of the [31]Si in the soluble fraction was precipitated by 5% trichloroacetic acid, suggesting an association with cytoplasmic proteins of at least a portion of the non-wall silica of *N. alba*. This material may be equivalent to Werner's PSA.

Sullivan (1979) used the [68]Ge-$Si(OH)_4$ radiotracer technique to evaluate the dynamic aspects of $Si(OH)_4$ metabolism, especially Si pool size and the kinetics of pool formation at various growth stages and during the synchronized division cycle of *Nav. pelliculosa*. These studies differ from the previous radiolabel experiments in that cells are grown for long periods of time (hours to 10 generation times) in the presence of [68]Ge-$Si(OH)_4$. Therefore the pools are fully equilibrated and the experiments are free of artifacts resulting from non-equilibrium changes in specific activity of radiolabel, and free of isotope exchange problems.

The $Si(OH)_4$ taken up by the cells entered at least two compartments: one extractable by 100 °C H_2O 15 min or 0 °C perchloric acid (PCA) 15 min, referred to as soluble Si pools, and the other, which was insoluble after extraction, called Si incorporation. The soluble and insoluble fractions by each

extraction procedure were quantitatively different; the H_2O-extractable pool was 40% to 50% larger than the PCA-extractable pool. However, the kinetic patterns of soluble pool formation were similar at most cell cycle stages. Again the difference between water extraction and acid extraction may represent a chemically unique fraction of silicon analogous to APSA as described by Werner (1966) and to the acid-precipitable fraction of the soluble supernatant described by Azam et al (1974). The H_2O and PCA-insoluble fraction is believed to consist predominantly but not exclusively of the silica frustule.

The soluble Si pools in *Nav. pelliculosa* were found to represent 9–13% of the total cell silicon during synchronized and exponential growth. Even after one or two generation times (12–24 h) under conditions of Si limitation the soluble Si pool sizes do not diminish significantly (Sullivan 1979). This some-what surprising result suggests that Si metabolism is very tightly regulated.

The Si pool size in fully equilibrated pools was extraordinarily high. The PCA-soluble and H_2O-soluble pool sizes are 490 ± 90 mM-Si l^{-1} cell water and 680 mM-Si l^{-1} cell water, respectively, for exponential cells and 438 ± 118 mM-Si l^{-1} cell water and 578 ± 134 mM-Si l^{-1} cell water, respectively, for synchronized cells. Since it is known from the physical–chemical studies of Alexander et al (1954) that solutions greater than 3.5 mM-Si(OH)$_4$ l^{-1} auto-polymerize by condensation to form silica gels or colloidal silica, it seems reasonable to hypothesize that the extractable Si pool contains, in addition to Si(OH)$_4$, one or more Si species as oligomeric silica chains or as colloidal silica macromolecules, $(SiO_2)_n$, possibly with molecular masses greater than 10^6 daltons. A scheme for this series is shown in Fig. 1. However the possible existence of organosilicon compounds in these pools has neither been con-firmed nor ruled out.
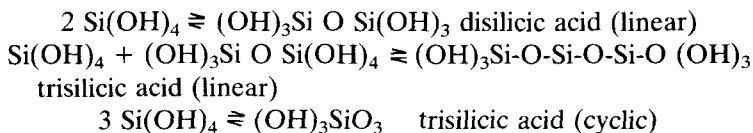
Chemical/biochemical modification of transported silicon

It is hypothesized that after transport and during Si pool formation the transported species is chemically modified to a polymerized form or to organo-silicon compounds (Fig. 1).

At least four possible chemical interactions are hypothesized to occur during the metabolism of Si(OH)$_4$ before it is finally polymerized into the silica valve. The products of these putative reactions may occur in the extractable pools. The first three reactions described below are drawn largely from the observa-tions of Greer (1971) during his studies of precious opal formation. While I am aware of the mineralogical differences between precious opal and biogenic opal of the diatom valve, several reports of colloidal-sized silica particles observed at actively silicifying sites in a variety of diatoms and other silicifying organisms (Simpson & Volcani 1981) suggest that these possibilities are worth careful investigation.

Condensation reactions of these types:

(1) *Low molecular mass silicic acids*

$$2 \; Si(OH)_4 \rightleftarrows (OH)_3Si \; O \; Si(OH)_3 \text{ disilicic acid (linear)}$$
$$Si(OH)_4 + (OH)_3Si \; O \; Si(OH)_4 \rightleftarrows (OH)_3Si\text{-}O\text{-}Si\text{-}O\text{-}Si\text{-}O \; (OH)_3$$
trisilicic acid (linear)
$$3 \; Si(OH)_4 \rightleftarrows (OH)_3SiO_3 \quad \text{trisilicic acid (cyclic)}$$

which result in polysilicic acids of relatively low molecular mass $\leqslant 8$ Si atoms/molecule. These molecules are considered as possible unstable or rapidly equilibrating intermediates in the diatom metabolism of Si, as described above.

(2) *Oligomeric polysilicic acid.* Three-dimensional polysilicic acids of from 8 to 20 Si atoms generally have less than two OH groups attached per Si atom, as proposed by Iler (1978) and Weiss (1978). These molecules would have molecular masses and characteristics intermediate between particle growth stages 1 and 2 in Table 2.

(3) *Colloidal silica particles.* Colloidal particles result from the aggregation of spherical polymers containing 40–50 or more silicon atoms, the outer surface of which consists predominantly of $(=SiO)_3$ SiOH groups that form hydrogen bonds because of their acidic nature. These possible intermediates in the soluble Si pools of diatoms are Si macromolecules of colloidal size range (1.5–60 nm) which may be analogous to those described by Greer (1971) and are shown in Table 2. Such colloidal particles may assemble to form spheres from 4 to 330 nm in diameter; and these in turn may form the immediate precursors of the silica valve. These particles are most likely to be present in soluble Si pools during the peak in pool size at the time of valve development, as shown by Sullivan (1979).

Silicon ligand complexes (chelates)

$Si(OH)_4$ is expected to form complexes with ligands such as organic hydroxy compounds, especially those with fixed diols as anion centres, where the $\begin{smallmatrix} C-O^- \\ | \\ C-O^- \end{smallmatrix}$ separation is about 0.26 nm and matches with the $Si\diagdown\begin{smallmatrix} O \\ O \end{smallmatrix}$ separation to form energetically favourable stable complexes. Candidates for such ligands include some sugars, tropones, unsaturated polyhydroxyl compounds, phenolates (for example 1,2-diphenols such as catechol species) and amino acids (serine and tyrosine) with two *cis*-OH groups, rigid structure and correct 'bite'. The

TABLE 2 Silica particle dimensions at various growth stages during formation of macromolecules and colloids

Particle growth stage no.	Observed diameter[a]	Radius	Calculated SiO_2		Particle molecular mass (Da) from (formula 3)	
			Sphere volume	Units/sphere		
	(Å)	(Å)	(Å³)			
1	2.65	1.32	9.60	1	60	60
2	15	7.5	1 765	183	1.10×10^4	1×10^4
3	100	50	523 125	54 463	3.26×10^6	3×10^6
4	600	300	1.12×10^8	1.16×10^7	6.96×18^8	7×10^8
5	3 300	1 650	1.87×10^9	1.94×10^8	1.16×10^{10}	1×10^{11}
6	20 000	10 000	4.1×10^{12}	4.26×10^{11}	2.77×10^{13}	3×10^{13}

[a] Greer (1971).

Estimated from: $V_{sphere} = \frac{4}{3}$, $r^3 = (4.185) (r)^3$, $Si(OH)_4 = 96g/M$, $SiO_2 = 60g/M$, O-Si-O bond length = 1.32Å.

(1) $VSi(OH)_4 = (4.185)(1.32 \text{ Å})^3 = 9.605 \text{ Å}^3$.

(2) $V_{\text{particle in units (SiO}_2)} = \dfrac{4.185(r)^3 = 0.4357(r)^3}{9.60 \text{ Å}^3}$.
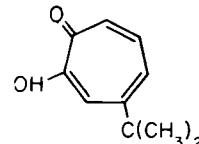
(3) $(V_{\text{in SiO}_2 \text{ units}})$ (60 g/mol SiO_2) = mol mass sphere $(SiO_2)_x$.

(4) Mol mass $= 26.14(r)^3$.

coordination of silicon may be 4 or 6 in these associations (see Sillen & Martel 1971 and Weiss & Herzog 1978).

Weiss & Herzog (1978) extracted from the evergreen *Thuja plicata* a Si–thujaplicine complex which represented as much as 15–22% of the Si in the green tips. The compound was identified as β-thujaplicine–silicon–hexa-fluorophosphate by X-ray and infrared spectroscopy.

β-thujaplicine has the structure:

Weiss & Herzog (1978) also postulate that the tannic acid–Si complex with its low solubility would allow a powerful accumulation of Si in tannin-rich structures in plants.

The Si ionophore described earlier would fall into this group of Si inter-actions with organic molecules.

Hydrogen bonding of Si(OH)₄ to mucopolysaccharides (glycosaminoglycans)

This type of interaction was demonstrated by Holt & Went (1960) to result in layered sheets of polysilicic acid which covered laminar films of a variety of chemically pure mucopolysaccharides and mucopolysaccharides from natural sources such as kelp. Hydrogen bonding of Si(OH) to protein/polysaccharides may play a role at membrane surfaces or at nucleation sites involved in diatom silicification, but none has been demonstrated.

Covalently bonded organosilicon compounds

Schwarz (1973) claimed the discovery of glycosamino compounds of high molecular mass containing Si atoms in covalent linkage through silanol bridges. The glycosamino–silicon subunit of these large molecules was however not isolated or purified. Similar activities may occur in diatoms but so far they have not been demonstrated. Why should one expect modified chemical species to exist within the soluble Si pools or in subcellular compartments of diatoms?

It seems reasonable to expect modification or compartmentation since silica, especially the polymerized form, has been shown to denature proteins and disrupt cellular membranes (Allison 1968). The diatom might, of course, have evolved protein structures and unique membrane properties which are not sensitive to silica denaturation or lysis, respectively. Barring this, alternative strategies might be used by diatoms for handling the large concentrations of silica required for valve development. For instance they might block the acidic oligomeric silica and colloidal silica surfaces by complex formation with the appropriate organic molecules. Otherwise they might exclude the colloidal silica particles from the cytosol by sequestering them in subcellular compart-ments such as Si transport vesicles, as mentioned earlier.

It is important to an understanding of Si metabolism to know the variety of chemical species present and the possible modes of their transformation. This is a current area of research interest in my laboratory.

Dynamics of Si metabolism leading to incorporation into a mineral product

The kinetics of $Si(OH)_4$ uptake, soluble pool formation and incorporation into insoluble material (silica valve) during the synchronized division cycle demonstrate the sequential pattern of these processes in Si metabolism (Fig. 2). Close inspection of the H_2O-soluble and H_2O-insoluble fractions during the first 0.5 h of addition of radiolabel to synchronized cells shows the rapid uptake of $Si(OH)_4$, of which 83% appears in the pool, while only 17% is incorporated. As
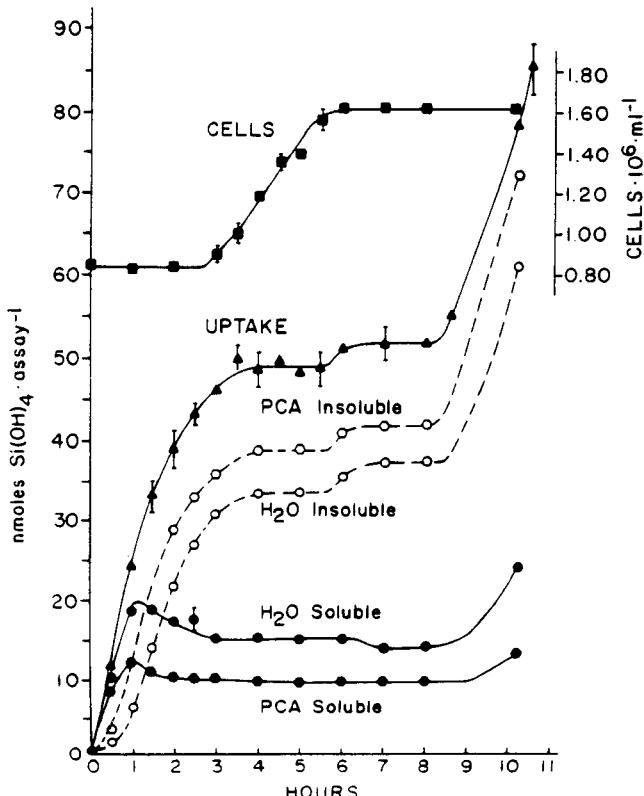
FIG. 2. Kinetics of $Si(OH)_4$ uptake, incorporation and soluble Si pool formation in *Navicula pelliculosa* during the synchronized division cycle. Cells treated by combined synchronization procedure: $^{68}Ge\text{-}Si(OH)_4$ mixture added to initiate development, resulting in a final concentration of 90 μmol $Si(OH)_4 l^{-1}$, 45 × 103 d.p.m. ml^{-1}, PCA perchloric acid. (From Sullivan 1979.)

the cycle progresses, the pools expand to a peak at 1–1.5 h, then contract; it is not until the pools contract, between 1 and 2 h, that the amount of Si incorporated exceeds that in the soluble pool. Although the significance of the peak pool expansion was uncertain (Sullivan 1979), its timing during the period of frustule formation suggests that it may represent the synthesis and intracellular build-up of the precursors of the silica frustule, as indicated above.

Thus far we have been able to 'visualize' the dynamic aspects of early events of silicon metabolism by using synchronized cells and a physiological/biochemical approach to understanding the problems of silicification better. The work of Chiappino & Volcani (1977) elegantly demonstrated by electron microscopic techniques frustule morphogenesis of the pennate diatom. *Nav. pelliculosa* and the reader should view the sequential growth of the valve presented there to gain a better idea of the coupling of physiological and morphological data sets. Through these combined techniques we have gained a better description of the sequential series of cell cycle events involved in the biomineralization process of the pennate diatom.

The autopolycondensation hypothesis

Background

Having described the kinetics of formation and steady-state pool sizes during exponential and synchronized diatom growth we could then explore several additional hypotheses about the biomineralization process which leads to silica valve formation in diatoms, and at the same time learn what are the energy and macromolecular synthetic requirements for silicification. The first, and possibly the most general, hypothesis to be tested was derived from the physical–chemical experiments of Alexander et al (1954) and Krauskopf (1959) which showed that at 24 °C supersaturated aqueous solutions (>3.5 mM) of monomeric $Si(OH)_4$ spontaneously polycondensed (autopolycondensed) to form amorphous or colloidal silica (SiO_2). At solubility equilibrium, a free $Si(OH)_4$ concentration of 3.5 mM remained in solution. This work was followed by studies of the growth of colloidal silica particles associated with precious opal by Darragh et al (1966) and colloidal assembly of the particles into larger aggregates by Greer (1971). Together they provided insight into physical–chemical processes of autopolycondensation and colloidal assembly which underlie formation of opaline silica; these processes may also have implications for the formation of biogenic opal, as in diatom valves.

The hypothesis to be tested was as follows. After transport of $Si(OH)_4$ to the cytoplasmic pools of the diatom, mineralization is a series of purely chemical reactions (autopolycondensation and colloidal assembly) which is not coupled to cellular metabolism.

This line of thinking implies that in order to effect autopolycondensation and

colloidal assembly the diatom would merely be required to fill its internal Si pool(s) to some threshold level by a single energy-requiring transport step. Once the threshold concentration was reached a series of chemical reactions uncoupled from cellular metabolism would be initiated which would lead to formation of the hydrated amorphous silica valve. A similar model has been proposed for silica deposition in higher plant shoots by Kaufman et al (1981). This hypothesis is also particularly attractive, since no elaborate metabolism for $Si(OH)_4$ would be required, nor would it be necessary to postulate the existence of specific protein templates (Hecky et al 1973) or silicase enzymes (Schwarz 1978) involved in the polycondensation reactions which result in the biogenic opal of the diatom frustule. As Darley (1969) points out, a mechanism such as this might also allow diatoms an energetically efficient cell wall synthesis.

Tests of the hypothesis

Considering the hypothesis of biomineralization of silica based on the physical chemistry of $Si(OH)_4$ autopolycondensation we can make three predictions which can be tested experimentally: (1) if purely chemical reactions can account for $Si(OH)_4$ polycondensation during the building of the diatom silica frustule, then polycondensation should proceed in cells where cellular energy metabolism is blocked; (2) threshold pool sizes (Si concentration) should trigger polymerization; (3) the intracellular pool concentrations of free $Si(OH)_4$ should be $\geqslant 3.5$ mM during active mineralization.

Evidence which is not compatible with the first prediction comes from several independent observations made during experiments with *Nav. saprophilia* presented by Sullivan (1980) and Blank et al (1986). Conditions which affect concomitant photosynthetic acquisition of energy (darkness and 3-3, 4-dichlorophenyl-1,1-dimethyl urea) have little or no effect on either biomineralization or cell separation (Fig. 3). These results suggest that:(1) photosynthetically derived energy is not directly coupled to biomineralization or (2) at the onset of silicification (post-cytokinesis) the cell possesses the necessary energy stores to complete the biomineralization process, including Si transport, intracellular translocation, polycondensation, colloidal assembly, valve formation and cell separation. However, as will be shown below, depletion of energy stores by agents that inhibit oxidative phosphorylation through disruption of mechanisms for maintaining proton gradients have an immediate effect on biomineralization (Sullivan 1980), thus indicating that energy is required for silicification. Using a different diatom, we found that six hours or longer of darkness altered the transport component of Si metabolism (Blank & Sullivan 1980). Short-term $Si(OH)_4$ transport after 15 min pretreatment with DCMU was reduced 50% relative to control transport rates (Sullivan 1976). Despite inhibition of transport to this extent, we have now shown in
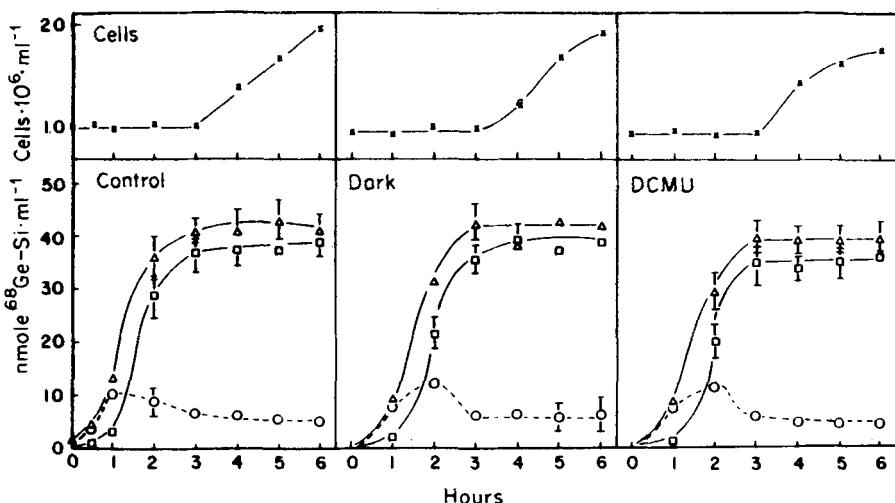
FIG. 3. Effects of dark and 3–3,4–dichlorophenyl–1,1–dimethyl urea (DCMU) on silicon biomineralization. DCMU was added, or cultures were darkened by being wrapped in aluminium foil, 1 h before the addition of silicon. Total uptake (Δ), incorporation ($\square$), pool size ($\circ$). (From Blank et al 1986.)

more detailed studies that DCMU has relatively little effect on long-term Si metabolism. Thus it appears that biomineralization is not directly coupled to concurrent activity from photosystem II or photosynthesis in general and must derive energy from storage material through substrate level phosphorylations.

As indicated in Fig. 4, the reactions or processes which underlie the incorporation of soluble Si into insoluble material in young synchronized cells are very sensitive to low temperature. It is clear that incorporation is even more sensitive to incubation at 0 °C than is the uptake of $Si(OH)_4$ from the medium an active process already shown to be intimately linked to cell metabolism, especially energy metabolism (Azam et al 1974, Sullivan 1976). Yet the low-temperature inhibition of Si incorporation is reversible and transfer of cold-labelled cells to a permissive temperature (20 °C) allows incorporation to proceed rapidly (Fig. 5a). A temperature-sensitive event (cellular process or reaction) post-cytokinesis is postulated to be required before silicification can occur in the pennate diatom.

When post-cytokinesis cells preloaded in the cold with $^{68}Ge-Si(OH)_4$ were incubated at otherwise permissive conditions but in the presence of 2,4-DNP, a specific inhibitor of oxidative phosphorylation, incorporation was completely blocked. The more general inhibitor N-ethylmaleimide (NEM) blocks thiol groups and NEM also completely stopped the incorporation of Si into insoluble material in both pulsed cells and pulse-chased cells (Fig. 5b).

These observations, taken together, suggest that silicification, at the step(s)
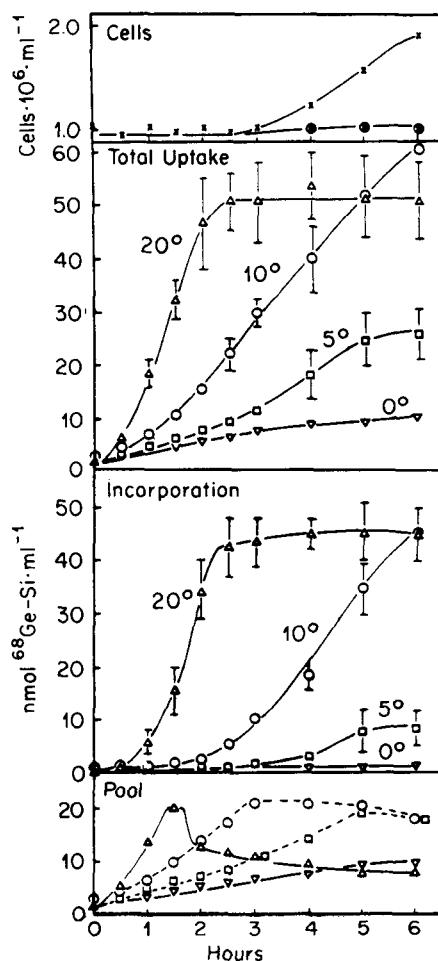
FIG. 4. Temperature effects on silicon biomineralization. $Si(OH)_4$ was added to synchronized cells 1 h after cultures had reached the indicated temperatures: 20 °C ($\triangle$), 10 °C ($\circ$), 5 °C ($\square$), 0 °C ($\triangledown$). (From Blank et al 1986.)

between intracellular pool formation and incorporation of Si into acid-insoluble and water-insoluble material, is not a purely chemical reaction. Rather, it requires ongoing cellular metabolism and specifically requires some form of energy which is available in the absence of concomitant photosynthesis, but is not available in 2,4-DNP-treated or NEM-treated cells. Thiol groups might be directly involved, or the inhibitory effects of NEM may, alternatively, be interpreted as a shutdown of the overall metabolism of the cell. These findings and conclusions are contrary to the ideas expressed by
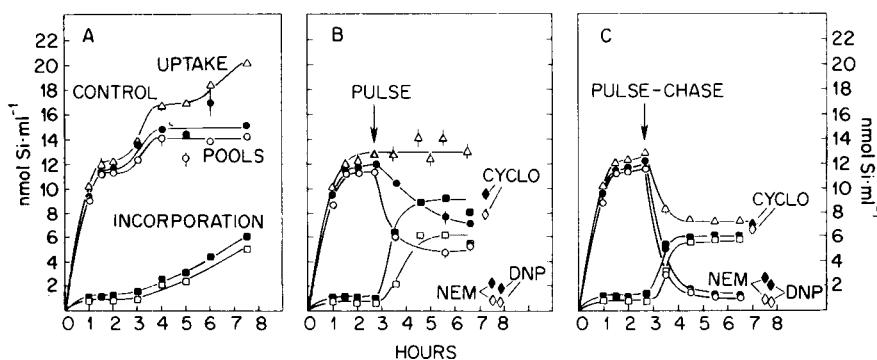
FIG. 5. ^{68}Ge-Si(OH)$_4$ pulse-chase experiments in cells preloaded for 3 h at 0 °C. (A) Control maintained at 0 °C throughout experiment. (B) Same as control but shifted to Si-free fresh water glycine medium (FWG) and permissive temperature (20 °C) at 3 h. (C) Same as control but shifted to FWG containing 100µmol Si(OH)$_4$ l^{-1} and permissive temperature at 3 h. Inhibitors were added at 3 h to subcultures of cells after resuspension but 10 min before the shift to 20 °C. Final inhibitor concentrations were: 10^{-3} M–DNP, 10^{-4} M-NEM, 10 µg cycloheximide ml^{-1} Symbols: uptake ($\triangle$); pools (○); incorporation (□). NEM, N-ethylmaleimide; CYCLO, cycloheximide. (From Sullivan 1980.)

Werner (1977) on the energetics of silicification.

The timing of synthesis of proteins required in silicon metabolism is shown by the effect of cycloheximide at various time points during Si metabolism (Fig. 6). Cycloheximide does not affect Si(OH)$_4$ transport rates on a short-term (60 s) basis (Sullivan 1976). Since some incorporation occurs when cycloheximide is added to young synchronized cells either simultaneously or 30 minutes later than Si(OH)$_4$, some of the putative biomineralization proteins, such as membrane proteins of the silicalemma or Si template proteins, must already be present. Addition of cycloheximide 1 h later than Si(OH)$_4$ results in higher levels of incorporation, but only with cycloheximide addition 1.5 or 2 h after Si(OH)$_4$ were the levels equal to the control level. Higher incorporation levels with later addition times for cycloheximide may reflect higher amounts of Si present in developing daughter valves, or more cells able to form new daughter valves, or both, and may result from (1) the increased presence of all the proteins required in silicon metabolism, (2) the presence of new proteins which are sequentially synthesized at discrete times during metabolism, or (3) the effects of an imperfect synchrony. Electron microscopic examination of developing valves taken later than 6 h after addition of silicic acid plus cycloheximide (at different times) shows developing valves at all time points of cycloheximide addition (unpublished work), suggesting either the presence of all proteins involved in Si metabolism at the time of cycloheximide addition or an imperfect synchrony. Blank et al (1986) found that the percentage of
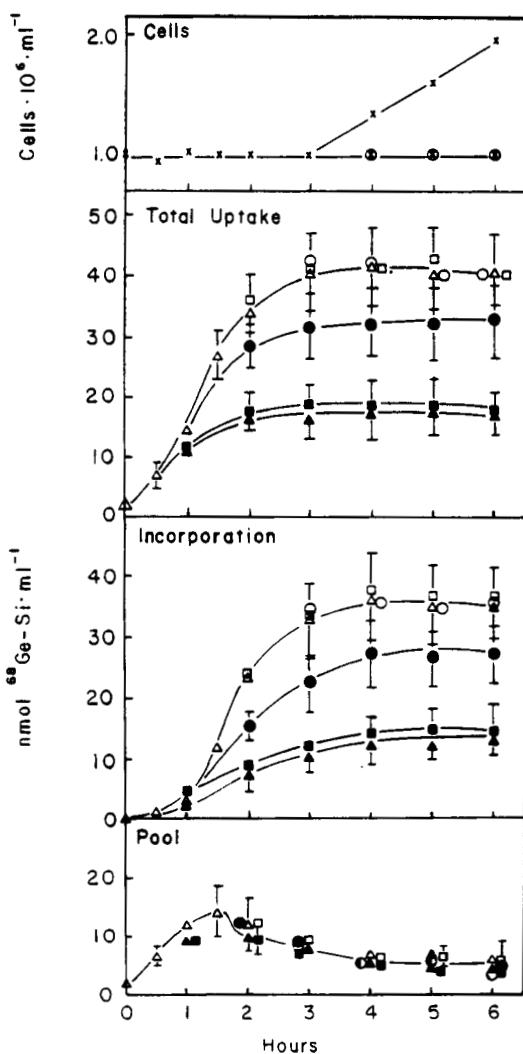
FIG. 6. Timing of cycloheximide addition: effects on silicon biomineralization. Cycloheximide was added to cultures at the indicated times to a final concentration of 10 μg/ml. Time of cycloheximide addition after silicon addition: no addition ($\triangle$), 0 h ($\blacktriangle$), 0.5 h ($\blacksquare$), 1 h ($\bullet$), 1.5 h ($\square$), 2 h ($\circ$). (From Blank et al 1986.)

aberrant valves first increases as the time of cycloheximide addition changes from 0 to 1 h and then decreases from 1 to 2 h after addition of silicic acid. The formation of lower percentages of aberrant valves with early times of cycloheximide addition may suggest that no valve formation occurs in some of these

cells. Although the synchronization procedure blocks cells in the same cell cycle stage (based on morphological development), one or more discrete biochemically defined substages may be present. This would result in cells in several substages with regard to biomineralization proteins at the time of silicic acid addition. These substages might be characterized as follows: (1) all proteins present permitting complete, normal valve formation; (2) some proteins allowing biomineralization but resulting in aberrant valves; (3) proteins required in biomineralization being absent and no valve being formed. The presence of synchronized cells in these substages suggests that most of the biomineralization proteins are synthesized either shortly before or just after cytokinesis. Although we were unable at this point to separate and identify the various proteins involved in silicon metabolism, the report of Okita & Volcani (1980) with a different diatom indicates that Si directly affects the expression of a small number of polypeptides (less than 10). Whether these polypeptides are those involved in silicon metabolism was not demonstrated. The presence of aberrations in newly formed valves may indicate that some of the proteins affected by cycloheximide are cytoskeletal proteins (Blank & Sullivan 1983).

Since cycloheximide addition up to 2 h after $Si(OH)_4$ addition completely blocked cell separation, the only proteins synthesized at a late stage of biomineralization are those involved in cell separation after valve formation. These proteins, possibly those involved in formation of the organic matrix surrounding the silica valve and any proteins which might be involved in the physical or chemical separation of the two daughter valves, are synthesized at a late stage after valve formation is nearly complete.

Other experiments, using preloaded cells to avoid reduction of Si transport rates caused by cycloheximide, revealed that de novo protein synthesis does not appear to be required for incorporation during the 3–4 h after cycloheximide addition to cells preloaded for 3 h and their incubation at permissive temperatures (Fig. 5b). By contrast, in cells preloaded for only 1 h, the addition of cycloheximide blocks incorporation during the following 2 h. This latter observation confirms that concomitant synthesis of protein(s) necessary for mineralization must occur during the 3-h period after $Si(OH)_4$ addition to synchronized cells (post-cytokinesis). These observations may have significant implications with respect to the macromolecular synthetic requirements of silicification. For instance, proteins thought to be essential for silicification that may be synthesized at this time are integral membrane proteins of the silicalemma, an organelle which apparently increases concomitantly with valve development (Chiappino & Volcani 1977), proteins of the silicon transport or silicon deposition vesicles, or the putative silicon template protein postulated by Hecky et al (1973).

In an effort to determine whether the incorporation of Si into the silica frustule depends on the Si pool concentration (size), we did pulse and pulse-chase experiments (Fig. 5). The results indicate that: (1) cells with incompletely

filled pools can incorporate at least a portion of the pool Si into acid-insoluble material, given sufficient time at 20 °C; (2) cells incubated at 0 °C build large soluble Si pools which may exceed the peak pool concentrations observed previously (Sullivan 1979), yet do not incorporate the Si into insoluble material, even during long incubation times.

Both observations suggest that some critical or threshold Si pool concentration is not likely by itself to trigger the polycondensation reaction which leads to silica valve formation; however, the results demonstrate that large Si pools are maintained in cells at all times, in confirmation of the previous observation of high pool levels even in synchronized cells starved of Si for 14 h (Sullivan 1979). One could argue that this rationale is flawed, since the [68]Ge–Si radioassay is an index of *total* Si and not monomeric $Si(OH)_4$. However, recent unpublished studies in my laboratory have shown that 48% of the water-soluble pool Si is reactive as a blue silicomolybdate complex and is monomeric $Si(OH)_4$. Such levels may still satisfy concentration-dependent conditions for silica polymerization.

Collectively these results constitute tests of the third prediction of the autopolycondensation hypothesis, that silicifying cells will maintain $Si(OH)_4$ pool sizes of $\geqslant 3.5$ mmol 1^{-1} cell water during the normal division cycle, and appear to confirm it by the three different experimental observations noted above. Thus, extremely high concentrations of intracellular Si pools appear to be characteristic of *Nav. pelliculosa*. This may be a general phenomenon, as one can see if one calculates the probable pool sizes in the diatoms investigated by Chisholm et al (1978). Note, however, that their method of determining Si solubility is rather severe, especially for lightly silicified species.

In addition to the direct testing of the autopolymerization hypothesis, several significant aspects of silicon metabolism were revealed in these studies. Total Si incorporation appears to be a three-phase event (Fig. 2). Kinetics reveal an initial 30 min slow incorporation followed by the major component ($\sim 95\%$) of Si incorporation into the developing valves at between 0.5 and 2.5 h. A minor component ($\sim 5\%$), lasting ~ 15–30 min, occurs between 3.5 and 4 h after addition of $Si(OH)_4$ to the synchronized cells. The latter component of incorporation is correlated with the timing of girdle band formation in *Nav. pelliculosa* and may, in fact, represent Si incorporation into this lightly silicified frustule component. This general pattern is consistent with the developmental and fine-structural evidence reported by others (Chiappino & Volcani 1977), as well as with previous physiological information (Sullivan 1979).

But we should pause here and ask an important question:

From these few species, can we now generalize this description and the outlined silicification model to centric diatoms (or for that matter other pennate diatoms)?

I think the answer is an unequivocal yes, based largely on the work of Pickett-Heaps et al (1979), Schmid (1985) and Li & Volcani (1985). Although

their ultrastructural work shows considerable morphological differences during frustule formation in several centric species, they all suggest common biochemical properties involving interaction of silicon with membranes such as the plasmamembrane, silicalemma, silica transport vesicles, silicon deposition vesicles, etc., and a role for microtubules, microfilaments and membranes in the shaping of the silica valve. However, this is not to say that important differences do not exist. The observation by Li & Volcani (1984) that in the centric diatoms the 'embryonic' silicified frustule formed before cytokinesis was complete has significant implications for silicon regulation during the cell cycle and calls into question the involvement of the microtubule organizing centre (Pickett-Heaps et al 1979) in silicification in these centric species.

Closing

In closing I would like to include a description of what Professor K. Simkiss has indicated is a 'generally accepted theory of biomineralization' in his provocative editorial note in Biomineralization Newsletter No. 4, 1981.

(1) Cells are involved in (a) energetic processes; (b) ion movements; (c) matrix secretion; (d) enzyme-mediated processes; and (e) shape determination of the deposits during the process of biomineralization.

(2) The sites of mineralization (a) are of controlled ionic composition; (b) exceed the solubility product for some mineral; (c) have nucleation or epitactic sites, or both; (d) depend on cellular activity; (e) are involved with specific enzymes; and (f) are free of inhibitors.

(3) The mineralized deposits are (a) chemically specific; (b) oriented crystallographically; and (c) deposited on a matrix (?).

This simple but elegant outline provides us with an excellent framework for designing experiments to test hypotheses which will substantiate or refute various ideas we have about the mechanisms which underlie biomineralization processes such as silicification and calcification, or the intimate participation of almost any mineral product within a living organism.

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DISCUSSION

Werner: In many plant systems there are no reliable marker proteins for the plasma membrane. In your cell fractionation scheme, how did you identify the plasma membrane?

Sullivan: We chose two membrane markers. One was a synergistically stimulated Na⁺/K⁺-transporting ATPase and the other was a 5′-nucleotidase in the presence of sterols and other biochemical characteristics of the membrane. Collectively that information suggested that the fraction was enriched in cytoplasmic membrane. There may not be a rigorous way of saying it is definitely a

plasma membrane-enriched fraction other than by using the tools available in general cell biology and applying them to the diatom.

Werner: Those two markers are also present in other cell fractions. This is not a special problem in diatom biology but for advanced cell membranes in general.

Richards: There was a drastic difference in uptake once you starved the diatoms of silica. There might therefore be some massive morphological change in the membrane, or some enormous chemical change that would allow that difference. Have you looked at this?

Sullivan: There is no obvious morphological change in those cells in the electron microscope. The affinity of the transport system increased fivefold and there was also a significant increase in the so-called V_{max}, the number of transport sites on the cell surface. The increase in the rate can be largely attributed to a combination of those two changes in the characteristics of the transport system. There may be other changes too which I did not measure. For example recent work by Bhattacharyya & Volcani (1983) has shown that diatoms have ionophores which are carriers of silicate and germanate across the cell membrane. It may be possible to show that their numbers may have been increased as well.

I was interested in trying to distinguish whether that 10-fold change in transport rate was a result of a derepression-type mechanism or an inducible system. It didn't seem to be either, in the sense that the 10-fold increase occurred whether silicon was there in the medium or not.

Richards: Do you know exactly what the silicon binds to and how tightly it binds? In those circumstances, when you come to break up the cell and have put in radiolabelled germanium, how do you know that it isn't shooting around all over the place and binding in a non-specific fashion?

Sullivan: I don't know what silicon binds to. There are two candidates. The transport system is a black box that may contain Na^+/K^+-ATPase (Sullivan & Volcani 1974). The ionophore may also be implicated in the transport system. However, the evidence for that is not complete.

Volcani: The organism we have worked with, *Nitzschia alba*, is an uncommon diatom. It is a heterotrophic organism which in the course of evolution has lost the chloroplast, and in this respect, and in some of its metabolic properties, it is somewhat similar to a mammalian cell. We can obtain beautiful clean vesicles of the plasma membrane with the capacity to take up silicate ions and so on. We have studied silicate transport in these vesicles in great detail so we feel pretty confident about that (Bhattacharyya & Volcani 1980).

Our detailed studies of synchronous or semi-synchronous silicon-starved cultures show normal uptake for the uptake involved in shell deposition (e.g. Coombs et al 1967). There is another uptake concerning silicon utilization for a whole variety of other processes (e.g. DNA synthesis, cAMP synthesis). We can show that the amount of silicon taken up is equal to the amount deposited in

the shell. I don't think that it is in any way defective. The system is quite reliable.

Werner: Other phycologists will not agree that *N. alba* is more of a mammalian cell. It is a diatom cell and it has no chloroplast. The problem is separating the plasma membrane from the Golgi and the endoplasmic reticulum and these are of course still the same in *N. alba.*

Volcani: The question is whether the marker enzymes which are conventional in other systems are similar to or different from those of diatoms.

Hench: If you expose silicon-starved diatoms to a high concentration of a soluble solution of aluminium or aluminium contaminated with silica, do you get irreversible changes in these mineralization processes? I know of no published account of the role of aluminium ion effects on diatom silicification.

Sullivan: I haven't done that.

Farmer: A plausible mechanism for the increased uptake after silicon starvation might involve a broad gateway, which represents the pump pushing $Si(OH)_4$ from the environment into a concentrated soluble pool of Si within the diatom, and a narrow gateway transforming this soluble reserve into opal. When the organism is starved, the soluble pool will be depleted and so provide a sink for the rapid uptake of $Si(OH)_4$ when it again becomes available.

Sullivan: Before doing the experiments I reasoned much like you did but I found that at the end of silicon starvation the pools are 75–80% full, as observed in exponential or synchronized cells. So your nice model cannot account for the observed behaviour of the transport rate.

O'Neill: In mammalian cells there are lots of systems for active transport of specific molecules. The sort of uptake you mentioned also happens with uridine, for example. There are energy-requiring specific active transport systems for all sorts of crucial metabolites which can be very rapidly substrate-activated over three orders of magnitude.

Sullivan: That certainly fits in with the findings I reported.

Daniele: Can you block the increase in the uptake rate by inhibitors of protein synthesis?

Sullivan: Yes. If we add cycloheximide to synchronized cells at 0, 1 or 2 h the transport rate never increases at all. If we add it as the synchronized cells are reaching that peak, the transport rate decays with a half-life of about 2 h. If we inhibit it at the peak or after the peak it decays with a half-life of about 1–1.5 hours. We are dealing with *de novo* synthesis or an insertion into the membrane.

Volcani: Dr P. Bhattacharyya in my laboratory studied in great detail the mechanism of transport of silicate ion in *N. alba.* The transport is driven by Na^+/K^+-ATPase and silicate is symported with Na^+, which is very similar to the phosphate transport (Bhattacharyya & Volcani 1983).

Williams: Has anybody made an affinity column for $Si(OH)_4$?

Sullivan: No.

Dr Volcani, did you mean that Na$^+$/K$^+$-ATPase was definitely the molecule that silicon bound to in order to be transported?

Volcani: No, silicate transport is coupled to the Na$^+$ gradient which is generated by the Na$^+$/K$^+$-ATPase system. It is a system similar to the system that has been shown for the Na$^+$-dependent amino acids and sugar transports.

Sullivan: Does it occur in freshwater diatoms as well as in marine diatoms?

Volcani: That remains to be looked at.

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Silica in higher plants

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Abstract. Opaline silica deposits are formed by many vascular (higher) plants. The capacity of these plants for silica absorption varies considerably according to genotype and environment. Plant communities exchange silica between soil and vegetation, especially in warmer climates.

Silica deposition in epidermal cell walls offers mechanical and protective advantages. Biogenic silica particles from plants are also implicated in the causation of cancer. Recent techniques are reviewed which may aid in the identification of plant pathways for soluble silica movement to deposition sites and in the determination of ionic environments.

Botanical investigations have focused on silicification of cell walls in relation to plant development, using scanning and transmission electron microscopy combined with X-ray microanalysis. Silica deposition in macrohair walls of the lemma of canary grass (*Phalaris*) begins at inflorescence emergence and closely follows wall thickening. The structure of the deposited silica may be determined by specific organic polymers present at successive stages of wall development. Lowering of transpiration by enclosure of *Phalaris* inflorescences in plastic bags reduced silica deposition in macrohairs. Preliminary freeze-substitution studies have located silicon, as well as potassium and chloride, in the cell vacuole and wall deposition sites during initial silicification.

1986 Silicon biochemistry. Wiley, Chichester (Ciba Foundation Symposium 121) p 90–111

Silicification of plants was first described by de Saussure in 1804 (cited by Jones & Handreck 1967). It occurs in the Pteridophyta, including the spikemosses, horsetails and ferns, and extensively in the Spermatophyta, composed of gymnosperms (including conifers) and angiosperms (flowering plants), subdivided into monocotyledons and dicotyledons. These groups include a wide range of silicified herbaceous and arboreal species (Parry et al 1984), the extent of which remains unknown.

Silicon is essential for normal growth of heavy accumulators such as field horsetail (*Equisetum arvense*) (Lewin & Reimann 1969). Newer techniques may elaborate the role of silica in a wider range of vascular taxa, encouraging greater recognition of its importance. Monocotyledons are heavier accumulators than dicotyledons, by a factor of 10 to 20 in grasses compared with legumes

(Jones & Handreck 1967). Lanning & Eleuterius (1983) found a similar result among 44 herbaceous plants, also observing that closely related species exhibited a similar absorption capacity. However, among monocotyledons, characteristic deposits occur in certain families but not in others. Also, silica uptake varies widely among species; for example, lowland rice accumulates more than dryland grasses (Jones & Handreck 1967). Dicotyledons also exhibit variation; some are heavy silicon accumulators, notably in the hemp and nettle families (Parry et al 1984). Thus, taxonomic affinity, genotype and ecological factors all interact to produce variation in plant silicification.

Ecological significance

Most terrestrial biomes (ecosystems) possess a silica cycle. Heavy accumulation by tropical vegetation results from higher transpiration rates, and from increased silica solubility in soils because of higher temperatures (Frey-Wyssling 1930). D. Piperno (cited by Rovner 1983) recently analysed 500 tropical rain forest plants and found 340, representing 40 different monocotyledon and dicotyledon families, which produced identifiable silica deposits. Silica occurs in wood tissue of several hundred tropical trees (Parry et al 1984). In tropical rain forests, most silicon may be in silicon-accumulator plants rather than in the leached lateritic soil (Carlisle et al 1977).

Dominant species of the temperate deciduous forest form characteristic wood and leaf silica deposits. A 15-year-old oak transpires 4.6 tonnes of water per year to produce 1.7 grams of silica phytoliths in the leaves (Bartoli 1979). Sugar maple forests (N. America) yield 90.1 kg/ha of particulate biogenic silica in foliage and wood (J.W. Geis, unpublished paper, AAAS symposium, 31 May 1983). Similarly, in the coniferous forest biome, red pine (*Pinus resinosa*) trees yield 42.3 kg/ha of silica in bark and bolewood (J.W. Geis, *op. cit.*).

Arctic-alpine plants reportedly contain little silica because soil solubility is reduced by freezing, and the growth period is short. However, silica deposits accumulate in shrub heathland vegetation, e.g. *Calluna vulgaris*, and plants of northern bogs where soluble silica may be higher in poorly drained, reduced soils (Frey-Wyssling 1930, Carlisle et al 1977, Bartoli 1979).

Silicification of submerged aquatic vascular plants is reported for only one family (Podostemaceae). However, some emergent fresh-water macrophytes, such as common reed (*Phragmites*), are heavy silicon accumulators (Frey-Wyssling 1930). Accentuated levels of plant silica might be expected from alkaline water bodies. Various coastal salt marsh plants contain silica in shoots ranging from 2 to 28% ash content, highest among grasses (Poaceae) and sedges (Cyperaceae) (Lanning & Eleuterius 1983).

Biomes of semiarid regions such as grasslands and savannahs typically exhibit plants of high silicon levels where soils may be alkaline and slightly leached (Carlisle et al 1977). Grassland species have been most intensively
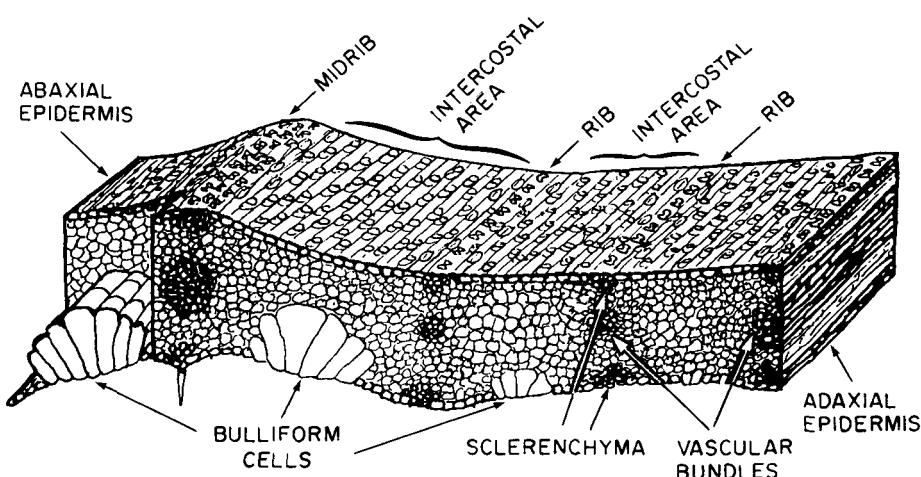
FIG. 1. Anatomy of the leaf blade of grasses, showing rows of epidermal silica bodies (opal phytoliths) and the location of internal tissues in cross-section. (Reproduced with permission of Dr D. Wynn Parry.)

studied (Parry et al 1984, Jones & Handreck 1967). L.G. Labouriau (cited in Rovner 1983) investigated the 'Cerrados' or arid region of Brazil, finding that not only grasses but also other characteristic vegetation types, including trees, contained diagnostic silica deposits.

Silica cycle

The involvement of vascular plants in this biogeochemical cycle includes root uptake, translocation and precipitation of silica in tissues, followed by the return of silica to soil horizons after death and decay. The biochemistry of the plants may also play a part in the cycle. Heavy accumulators (e.g. *Equisetum*) may release root exudates which depolymerize soil silicates. Thus, silica absorption can exceed that normally available in soil water. Humic acids from decay may increase silicate dissolution in soils of high organic content. Active root uptake and transport of silicic acid are reported for rice, based on studies of metabolic inhibitors. However, in oats, silica uptake is explained by passive diffusion. Dicotyledons may actively exclude silica at the root surface, or internally. Silicon accumulators such as rice or sugarcane change from active absorption at the root surface to active exclusion as the ambient silicon concentration rises. Silicic acid in upward flow towards the leaves may exhibit supersaturated levels in xylem sap, e.g. 650 p.p.m. SiO_2 in rice. Polymerization may be prevented because Si–OH groups of silicic acid are complexed with organic substances such as phenols. At deposition sites, silica may complex

with cell wall polymers (e.g. sugars) (Lewin & Reimann 1969, Birchall 1978, Parry et al 1984).

Location and function of silica deposits in grasses

Amorphous silica deposits occur in (1) cell lumens, (2) cell walls, and (3) intercellular spaces or external layers (Sangster & Parry 1981). Jones & Handreck (1967) determined that the highest levels of silicon in cereals occur in the inflorescence, followed by the leaf blade and sheath, the culm (stem), and the subterranean organs, from which they inferred a passive mechanism of deposition. However, McNaughton et al (1985) have reported the opposite for perennial African grasses in which silicon content diminishes from the root upwards. Thus, they reject the concept equating maximum silica accumulation with the most active evaporation surfaces for these grasses.

In shoots, the epidermis is the primary deposition site, especially in hair (trichome) walls and lumens of silica cells (idioblasts) forming opal phytoliths, in rows above leaf veins and margins, or else scattered between veins, or over culm internodes. Subsequently, walls and lumens of other epidermal elements including long cells, bulliform cells and stomates may become silicified, as well as internal mesophyll, conducting and strengthening (sclerenchyma) cells (see Fig. 1).

Silica deposits in roots occur internally in the endodermis and conducting system or in the epidermis. In the rhizome (underground stem), epidermal deposition prevails, followed by vascular-associated deposits. Air canals, if present, are silicified in older rhizomes.

The extent of silicification is influenced by developmental anatomy. For example, in roots, which exhibit a longitudinal gradient of tissue maturation, silica deposits are confined to the older three-quarters of the root, being absent from the extending tip region. In growing tissues, deposition sites are very localized, exhibiting functional significance as in the rhizome apical epidermis, but deposition sites in older organs may become increasingly non-specific (Sangster & Parry 1981, Parry et al 1984, Sangster 1985).

Many functions have been ascribed to silica deposits in plants (Jones & Handreck 1967, Lewin & Reimann 1969); these functions are generally classified as structural, physiological or protective, and they are frequently interrelated. Silica contributes compression–resistance (Raven 1983) and rigidity in cell walls, which aids photosynthetic efficiency by improving light interception (Bartoli 1979), improves drought resistance, and reduces lodging in cereal stems. Physiological functions include curtailment of evapo-transpiration and promotion of oxygen supply to rice roots owing to strengthening of the air canals. Protective functions include resistance to pathogens, insects, molluscs and grazing by herbivores. Silicified inflorescence bracts inhibit consumption of seed tissues, and deposition removes excess, possibly

toxic, levels of silicon (Kaufman et al 1981, Parry et al 1984). Thus, structural silica positively reinforces normal growth, development and yields, while its protective function relates to the evolution of grass–herbivore interactions.

Current investigations

Much current research concentrates on techniques for identifying opal phytoliths from soils and archaeological sites; these techniques are useful in determining ancient climates (palaeoecology), the origin of cultigens, and human cultural prehistory (ethnobotany) (Rovner 1983).

Phytolith distribution patterns over leaf and stem surfaces of the Poaceae reveal important palaeobotanical and evolutionary implications. Grasses may be divided into C_3 and C_4 groups depending on whether they exhibit one of two photosynthetic biochemical pathways. C_3 grasses originate from temperate cool regions, while C_4 grasses typically grow in warmer climates. Phytolith shapes produced by C_3 grasses (e.g. circular) are distinguishable from those of C_4 grasses (e.g. cross-shaped); thus, recovery of these identifiable phytolith types from soils aids in palaeoclimatic reconstruction (P. Twiss, unpublished paper, AAAS symposium, 31 May 1983). However, among the Panicoid grasses climatic reconstruction is complicated by the presence of C_3 as well as C_4 species, which resemble each other in their phytolith shapes (D. Wynn Parry, personal communication).

Kaufman et al (1981, 1985) analysed leaf epidermal phytolith systems. They demonstrated a higher frequency of silicified sites, notably idioblasts and bulliform cells, in C_4 grasses as compared to C_3, but found that silicified trichomes (hairs) occur with greater frequency on C_3 leaves. They reject their previous 'window hypothesis' which suggested that silicified cells facilitated light transmission to internal photosynthetic tissue, finding rather that light was reduced.

Our investigations (in association with Dr D. Wynn Parry, School of Plant Biology, University College of North Wales) have centred on silicification in plant cell walls and lumens, emphasizing the integration of biomineralization with plant developmental stages, and environmental factors.

Recent advances in techniques

Sangster & Parry (1981) reviewed the light microscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM) and electron-probe microanalysis (EPM) techniques for detection and analysis of deposited plant silica.

Recently there has been renewed interest, in work with light microscopy, in using specific stains such as methyl red, crystal violet lactone and silver-ammine chromate for silica bodies; these may indicate the surface chemistry of phytoliths (Kaufman et al 1985). These stains, combined with computer-

programmed, soft X-ray image analysis, were used to determine silica body distribution and frequency in grasses.

The analysis of soluble silicon in xylem exudate has proved to be difficult as the volumes of exudate are too small for standard procedures. Recently, Gartner et al (1984), using a refinement of EPM techniques, have determined silicon concentrations in small samples ($<$ 1 nl) of xylem sap from wheat. Similar techniques might be used to estimate silicon concentrations in phloem sap and cell vacuoles.

Proton-induced X-ray emission (PIXE) combined with a scanning proton microprobe was used (Perry et al 1984b) to investigate the distribution of inorganic elements, including silicon, in the lemma macrohairs of canary grass (*Phalaris canariensis*). PIXE is more sensitive than EPM, having low background bremsstrahlung radiation.

Among the major problems now facing investigators are identification of the pathways by which soluble silica reaches deposition sites and ionic analysis at these sites. New cryo-techniques in electron microscopy may be helpful here. The two most promising techniques are scanning X-ray microanalysis of bulk-frozen hydrated tissue and transmission X-ray microanalysis of freeze-substituted sections (Harvey 1983). (The latter technique will be discussed in the section on *Phalaris*.)

Wheat epicarp hairs

Silicification of the epicarp hairs of wheat, *Triticum aestivum*, cultivar Highbury (Fig. 2), was described by Parry et al (1984). Most silica was deposited as a thin (100 nm) outer wall layer shortly after inflorescence emergence. Increased transpiration from the hairs at this time may proportionately increase deposition. If so, epicarp hairs from an arid environment might contain more silica than those from temperate climates. To test this hypothesis, we analysed mature hairs from eight cultivars, including five from arid Mexican regions (Table 1). Bases were lower in silicon than the rest of the hair. Considerable variability in silicon content, both within cultivars and between them, was apparent. Climatic-related trends are difficult to discern from the X-ray data obtained. Hair silicification is probably determined by an interaction between climate and wheat genotype.

Plant silica and cancer

The possible involvement of biogenic silica in human cancer has been reviewed by Parry et al (1984). Silica particles from inflorescences of *Setaria italica* and *Phalaris* species may promote oesophageal cancer in localized regions of China and Iran, respectively. Bhatt et al (1984) showed that mature macrohairs from the lemma of *Phalaris canariensis* act as skin tumour promoters in the
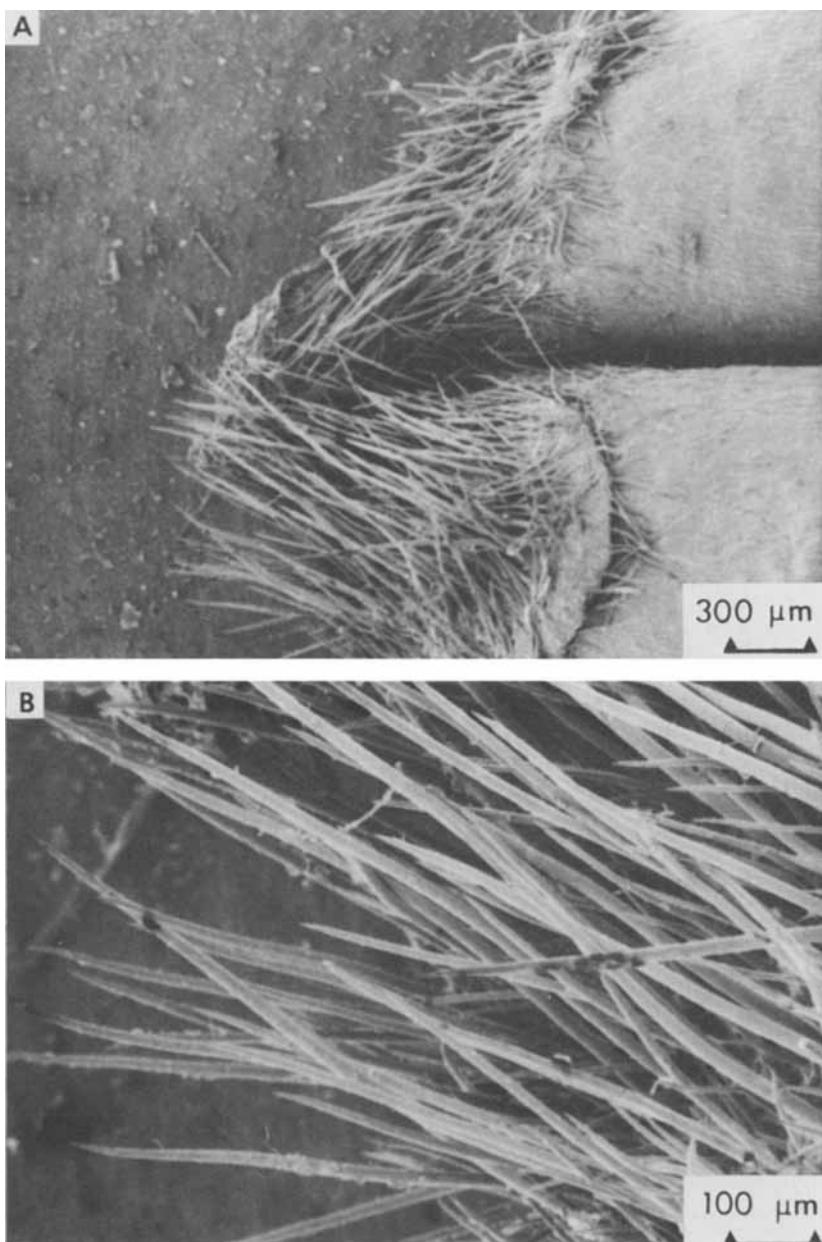
FIG. 2. Scanning electron micrographs illustrating epicarp hairs at the apex of a wheat grain (*Triticum aestivum* L. cv Highbury). (A) Mature brush end of grain showing a profusion of epicarp hairs. (B) Higher magnification of epicarp hairs.

TABLE 1 Comparisons of levels of deposited silicon in the epicarp hairs of eight wheat cultivars

| Cultivar | Origin | Climate | Analysis position on hair[a] | | |
			Base	Middle	Tip
Yecora	Mexico	Arid	7693 ± 2256	9 269 ± 2045	10 314 ± 3639
Glennson	Mexico	Arid	6015 ± 2358	12 653 ± 5080	10 798 ± 5496
Seri	Mexico	Arid	1774 ± 629	7 438 ± 2360	7 613 ± 2840
Ures	Mexico	Arid	3305 ± 4292	11 112 ± 9202	8 691 ± 8003
Genaro	Mexico	Arid	9902 ± 7519	12 208 ± 8921	6 436 ± 4245
Norstar	W. Canada	Semi-arid	2155 ± 485	4 991 ± 1796	5 593 ± 1574
Gordon	E. Canada	Temper-ate	611 ± 501	2 229 ± 1461	4 532 ± 3226
Highbury	United Kingdom	Temper-ate	2418 ± 1061	10 953 ± 5144	11 441 ± 3731

[a] Number of X-ray point counts for 10 s on the scaler timer of an electron-probe microanalyser peaked for silicon (K_α, 1.739 ke V) (mean and standard deviation, $n \geq 18$). Overall background level of Si, 33 ± 29 ($n = 12$). Pure silicon wafer standard, $164\,081 \pm 6231$ ($n = 5$).

presence of polycyclic initiators in test mice.

In bracken, a pteridophyte known to contain organic carciongens affecting cattle, the major silica deposition site is the outer tangential walls of the petiole epidermis. In addition, long siliceous fibres are associated with the leaf midribs. These fibres may act synergistically with the organic carcinogens (Parry et al 1985).

Silica deposition in Phalaris canariensis

Review of previous studies

Inflorescence bracts of *P. canariensis* provide a useful system for the study of silica deposition, especially the lemma with its unicellular macrohairs covering the outer epidermis (Fig. 3A, B). Sangster et al (1983) used light microscopy, SEM and EPM to observe lemma development from two weeks before inflorescence emergence to inflorescence maturity, some six weeks afterwards. Major sites of silica deposition are the outer epidermal cells and macrohairs, and deposition begins immediately after emergence. Further information on macrohair development has been provided by Perry et al (1984a,b) who showed, using SEM, TEM, energy dispersive X-ray analysis and PIXE, that the immature macrohairs contain potassium, phosphorus, sulphur and chlorine,
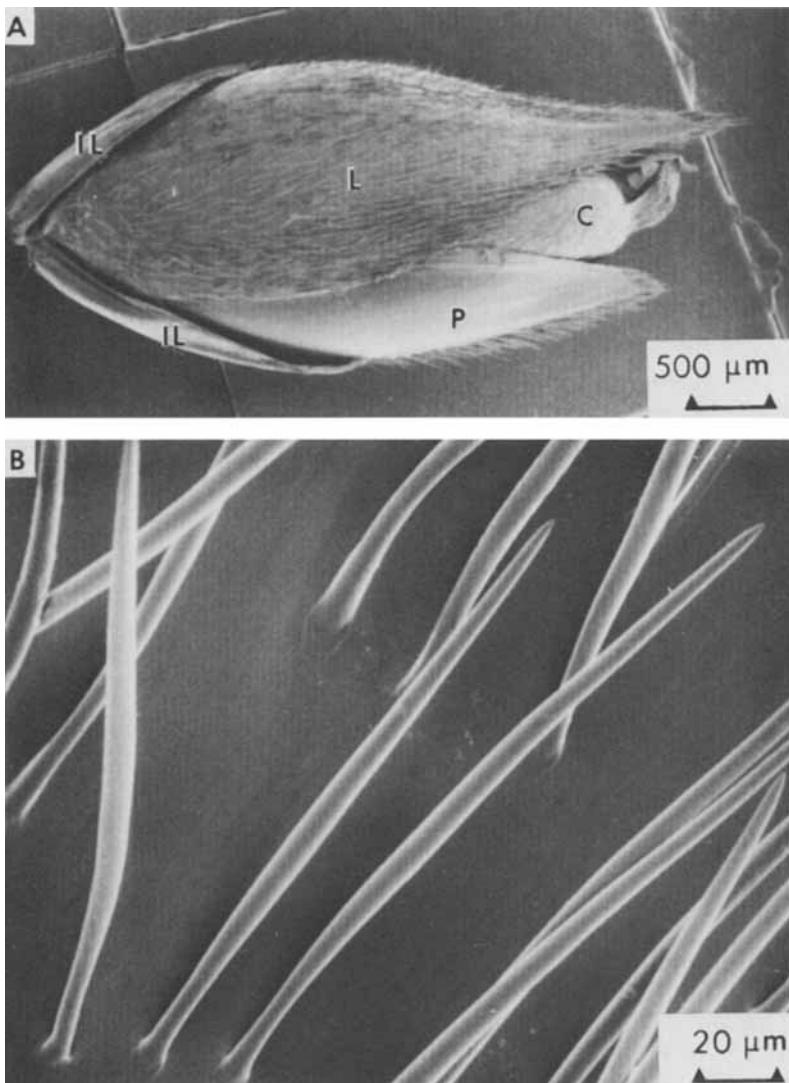
FIG. 3. Scanning electron micrographs illustrating some features of the florets of *Phalaris canariensis* L. (enclosing glumes removed). (A) Group of three mature florets. The single, fertile floret exhibits a lemma (L) and palea (P) enclosing the caryopsis (C). The basal bracts are infertile lemmas (IL) representing two sterile florets. Note macrohairs covering the lemma and part of the palea. (B) Macrohairs above the epidermis of a mature, fertile lemma.

but little or no silicon. Conversely, mature hairs are highly silicified, but have much lower levels of the other elements. Hodson et al (1984) studied macrohair ultrastructure and showed that silica is first deposited in the outer cell wall shortly after inflorescence emergence. During the next two weeks, the macrohair wall thickens considerably, leaving a narrow central lumen, and silica is eventually deposited throughout this wall. Mature macrohair transections reveal a series of concentric wall layers. Perry et al (1984a) found that silica deposited in these layers exhibits three different physical forms. This implies that the deposition environment differs at various times during macrohair development.

The glumes of *P. canariensis* have a more complex epidermal anatomy than the lemma, exhibiting macrohairs, prickle hairs, papillae and silica-cork cell pairs. Unlike the lemma, many of these cells become silicified before inflorescence emergence (Sangster et al 1983, Hodson et al 1985). Various questions arise from the developmental work. The following sections present preliminary experimental findings which may help to provide some answers.

Wall composition

The cell wall is an important organic matrix for silicification, but this matrix has only recently been analysed. Waterkeyn et al (1982), using specific staining techniques, showed that callose formation precedes silicification in trichome walls of various species. The cell wall of lemma macrohairs of *P. canariensis* has been analysed at several developmental stages by Perry (1985). Cellulose and arabinoxylans are the major constituents of young hairs, but later in hair development non-cellulosic glucans are more prevalent. These differing carbohydrate substances constituting layers of the macrohair wall may impose a different structure on deposited silica within each layer, as observed by Perry et al (1984a).

Does transpiration affect silicification of lemma macrohairs?

Silica deposition begins in the lemma macrohairs immediately after inflorescence emergence (Sangster et al 1983). Thus, atmospheric exposure and increase in transpiration from the lemma might influence silica deposition. High humidity is known to reduce transpiration. In a simple experiment (M. J. Hodson, unpublished) inflorescences were enclosed in plastic bags on emergence, raising the humidity around the inflorescence considerably. Plants were grown in a growth cabinet in water culture containing 50 p.p.m. SiO_2, as described by Sangster et al (1983). Inflorescences emerged after six weeks. Some were bagged immediately, while controls remained in the ambient environment (18 h photoperiod, 25°C, 70% r.h., alternating with 6 h darkness, 15°C and 61% r.h.). Replicate lemmas from both treatments were harvested at
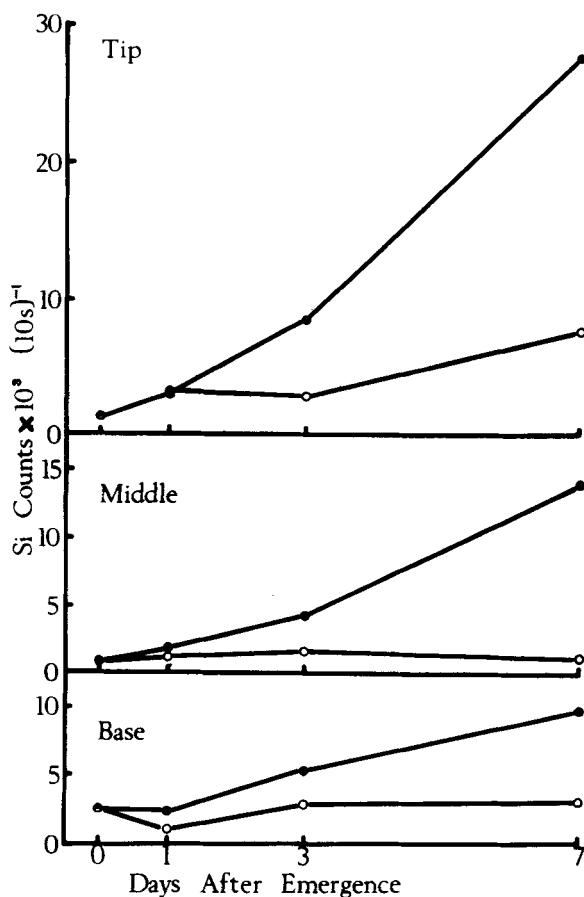
FIG. 4. The effect of humidity on silicon deposition in lemma macrohairs of *Phalaris canariensis* L. Graphs illustrate the mean number ($n \geqslant 18$) of X-ray point counts for 10 s on the scaler timer of an electron-probe microanalyser peaked for silicon (K_α, 1.739 KeV) from hairs at tip, middle and base stations on the fertile lemma. Silicon standards as for Table 1. Harvests were taken up to one week after emergence. -●-Control inflorescences, allowed to emerge into growth-cabinet environment (see text).-○-Inflorescences enclosed in plastic bags at emergence.

0, 1, 3 and 7 days after emergence and prepared for silicon analysis by EPM. Macrohairs were analysed from tip, middle and base stations on the lemma. Pooled data are illustrated in Fig. 4.

In the controls, silica deposition rate during the week after emergence was greatest in macrohairs from the tip region and least for basal hairs. Those from the middle station showed intermediate values. Tip hairs are also the first to extend above the outer epidermis (Sangster et al 1983), demonstrating a

developmental gradient running from tip to base of the lemma. Increasing the humidity by using plastic bags at emergence reduces silica deposition at all three stations. This experiment invites the criticism that bagging not only increases humidity but may also upset oxygen and carbon dioxide levels around the inflorescence. However, bagging probably represents a close approximation to the enclosed natural environment of the inflorescence before emergence. Deposition in lemma macrohairs may be influenced by transpiration but this does not preclude active metabolic processes from also being involved.

Pathways and deposition conditions

Previously, deposited silica was located in the lemma macrohairs of canary grass by electron microscopy and X-ray microanalysis (Sangster et al 1983, Hodson et al 1984). The timing and sites of silica deposition were elucidated but not the pathways by which soluble silica moved to deposition sites. These techniques do not retain soluble components within plant tissues. Two possible routes for silicon movement in macrohairs, not mutually exclusive, are through the cytoplasm and vacuole, or along cell walls. Perry et al (1984a, b) detected Si, K, P, S and Cl in macrohairs but not their precise location, and no precautions were taken to restrict the movement of soluble components. To elucidate silica deposition mechanisms, all components at deposition sites, including soluble ions, must be identified.

In preliminary studies (M. J. Hodson & A. Bell, unpublished) we used freeze substitution, which retains soluble ions in their *in vivo* locations. Before emergence, K and Cl are present in the macrohair vacuole, cytoplasm and wall. Three days after emergence, electron-opaque siliceous material is deposited in the outer wall layers; K and Cl are also present here. In addition, Si, K and Cl were detected in the non-silicified wall area. The cytoplasm and vacuole generally contain much K and Cl and little Si, but in a few hairs a considerable amount of Si was detected, presumably soluble. Five days after emergence more silica was deposited in the cell wall, with other ions as before; Si was not detected in cell vacuoles. At all harvests phosphorus was present in cytoplasm and nuclei, with less in the vacuole.

These preliminary findings may suggest that the cell wall is an important silicon pathway within the macrohair, which supports the hypothesis that silicon is transported passively by transpiration. Vacuolar silicon in a few macrohairs three days after emergence may represent a transient stage brought about by increased post-emergent influx of silicon (Fig. 4). However, although vacuolar silicon levels are low during macrohair development, silicon transport via the protoplast could be explained by rapid export rates to deposition sites, matching import rates. Thus, an alternative active-transport mechanism may also be involved.

Only K and Cl occur in significant quantities at wall deposition sites. High

chloride levels result from the nutrient solution chosen. Thus, potassium is the most likely ion to influence silica deposition in cell walls of this system under field conditions.

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DISCUSSION

Werner: You said that when transpiration was reduced, deposition of silica in the macrohairs was also reduced. Did you calculate the concentration of silica in different solutions in relation to total water movement through the plant, or even through the hairs? Would this tell you whether the space in the epidermal cells is just enough to deposit all the silica there? Or would it perhaps show that there is not enough space to deposit all the silica, yet a certain amount is continuously excreted, which we don't see because it is blown away from the surface?

Sangster: Dr Hodson performed the experiment simply to see whether the method would affect deposition in the macrohairs. No measurements of the type you are referring to have been done yet. Transpiration from the macrohairs is difficult to measure. We don't even know if they are transpiring. The mineral was initially deposited around the outside of the hair and one wonders whether this blocks water loss to the outside. It is unlikely that much solid silica is blown from the epidermal surface.

Werner: It is very difficult to measure this in the hairs, but have those calculations been done for total leaves?

Sangster: This could be more easily done. However, in the *Phalaris* study described, the macrohairs were located on the outer surface of minute inflorescence bracts. All the stomata are on the inner surface so there is no water

loss from this source on the exposed surface, yet enclosing the inflorescences in plastic bags reduces the amount of deposition. There is a problem here of distinguishing the transport of silica from its deposition. Are the bags interfering with deposition, although they do not stop it, or are they just depressing the transport of silica to those sites so that there is not as much available to be deposited? The question may only be resolved using freeze substitution. It clearly relates back to the problem of distinguishing between soluble silica and deposited silica.

Parry: The plastic bags would delay maturity of the bracts and directly affect the deposition mechanism.

Sangster: The inflorescence study does indicate that even though you interfere with the transpiration rate you still get some deposition.

Farmer: Jones & Handreck (1967) looked at the volume of water transpired, the concentration of silica in the soil solution, and the amount of silica that accumulated in grass. They found a very good balance, which they thought was evidence for a passive uptake of silicic acid.

Williams: Then one would have to say that other species deliberately excluded silica. Most people would believe that there are exclusion mechanisms in plants. Are there also positive uptake mechanisms at the root level?

Sangster: There are plants where passive uptake does not apply. Rice, for example, is an effective accumulator of silica, so investigators have concluded that it exhibits both active uptake and deployment of silica.

Werner: The experiments by Jones & Handreck were done with relatively high concentrations. For active uptake the silica concentration should go down at least three orders of magnitude. There is active uptake with low concentrations and another non-active uptake with higher concentrations. This might be similar to succinate uptake in rhizobia, for example, where two transport systems have been found with a K_m different by orders of magnitude.

Parry: Some Australian workers have correlated the amount of silica deposited as a measure of transpiration (Hutton & Norrish 1974). There is a very close correlation.

Sangster: Van der Vorm (1980) found that several cereals were active accumulators of silica at low concentrations in the external root media, but that active uptake decreased progressively as the external concentration increased. At high concentrations, the same plants became active excluders of silica.

Hench: You mentioned that in rice organically fixed silica is thought to bond to phenol hydroxyl compounds. Could you expand on that?

Sangster: Such bonding might possibly account for the supersaturated levels of silica in the upward-flowing sap of stems as measured for some plants. Weiss & Herzog (1978) isolated a tropolone–silicon complex from a conifer, remarking that dihydroxyaryl compounds are strong chelating agents for silicon. The complex could decompose, releasing silicic acid at the deposition site. However, the quantity isolated was small, and the evidence for its plant origin was in

some doubt.

Perry: A report from Reading disputes that work. For the same plant system the Reading group were not able to extract the complex which Weiss said existed (Peggs & Bowen 1984).

Hench: How is the high concentration maintained in certain plants?

Sangster: There appear to be two possibilities. One is related to whether or not root uptake is active or passive, and the other to whether organo-silicon complexes are involved in transport. Hartley & Jones (1972) concluded that there was no evidence of any organo-complexing of the silica in plant sap. Compounds fractionated from the xylem exudate behaved solely as silicic acid. Hartley & Jones doubted that the earlier infrared spectroscopy studies suggesting that there was complexing with phenols were reliable. Most subsequent work on higher plants seems to be in agreement with the position.

Williams: Dr Perry made some of the compounds we are talking about. They are stable in water at about pH 10, but they are not stable in water at pH 7.

Perry: There is a certain amount of disequilibrium between the octahedral silicon complexes and uncombined silicic acid, even down to pH 7.8–7.9. Below this, the complexed silicon species dissociate completely.

Williams: These particular silica complexes are relatively easily hydrolysed, so they are not likely to be used in a transport mechanism at pH 7, but that does not exclude some special trick of synthesis where such phenolate groups are linked in a designed framework. Nobody has tested a special cavity designed to carry Si.

Parry: How therefore would you explain the deposition of opaline silica in intercellular spaces?

Williams: The only way I could explain that is to say that the SiOH surface has been very highly stabilized by an organic matrix. Otherwise a very high concentration of free silica is needed for it to precipitate, which is not known to occur. The solubility of opals at the beginning of deposition is very high—they are non-crystalline, so the question is how can a high enough concentration be built up to get it to precipitate at all. The only way I can think of is by generating a very highly hydrogen bonded and stabilized surface. The whole system is stabilized in favour of precipitation through the surface attachment. That does not require a covalent carrier mechanism using the phenols, though.

Hench: I asked that question because we have been studying the role of organic additives in controlling the rates of hydrolysis and condensation reactions in metal organic-derived silica gels (Hench 1986). Hydroxyl species are needed to shift the rates of condensation and hydrolysis in our laboratory studies. Maybe there is some evidence that biological condensation rates are shifted in the presence of such species as well.

Williams: Saturated dihydroxy compounds do not give silica compounds at all readily, as far as I know. In some ways that is surprising. With boron one gets to the condensed compounds very readily. Aluminium and iron chemistry also

react. The problem with Si may well be something to do with the difficulty of making bond angles to fit the size of this atom. Biology may be clever enough to rig the bond angles and use, say, a five-membered ring rather than a six-membered ring, to alter the bite of the OH groups. If it can find the right bite it could make SiOH condensates but nobody has made such a compound yet.

Volcani: The diatom has made it. We tried to dissect and understand what the so-called 'silicate pump' consists of. Dr Bhattacharyya found that two compounds of low molecular mass reside in the plasma membrane of the diatom *Nitzschia alba*; they are bound to protein and appear in minute quantities. We inserted these molecules into liposomes, i.e. small vesicles of lipids, and found they facilitate silicate transport across the membranes. The binding affinity of these ionophore-like compounds is very high. We have not yet elucidated the structure of the compounds. They have a diol residue which may be interacting with silicate.

Birchall: A whole mass of simple organic molecules, starting with methanol and working up through glycerol and so on, all have enormous effects on the rates of polycondensation of silicic acid and quite concentrated solutions remain clear and free from solid. This may suggest that a compound is formed. But this is misleading. There are just hydrogen-bonded complexes which slow down but do not stop polycondensation. It is not specific. Methanol and ethanol do this and glycerol and polyhydroxy compunds have even stronger effects. Iler (1979) gives a list of hydrogen bonding agents in order of activity.

Hench: We have found very specific effects with the metal organic-derived gels. Some in that series have no effect in controlling large-scale structural organization, and others do. We don't know why. We can get structures as large as 40 cm^2 in ambient temperatures that don't break and crack up.

Perry: An important distinction must be made between the transportation of silicon compounds in solution and the deposition of the solid mineral phase. Deposition is not necessarily involved with organic molecules in solution, but may be involved with the same molecules incorporated into or on a solid substrate. Binding properties and structural properties of molecules may be substantially different, depending on whether they are found in solution or at a solid surface. There are two distinct problems.

Williams: We should distinguish the carrier mode from the deposition mode.

Carlisle: A rich source of silicon in the diet is dietary fibre. Large-scale studies in both Canada and the United States have found a beneficial effect of adding generous amounts of rolled oats to the diet of patients with maturity-onset diabetes. Rolled oats are relatively high in silicon content compared to other commonly eaten cereals and it is possible that some of the reported beneficial effects may be due to their high silicon content. Can you say anything about the structure of silicon in plant polysaccharides such as polygalacturonic acid?

Sangster: In oats, the floral bracts (husks) remain attached to the grain. This

would explain the higher silica content, because the inflorescence bracts generally represent the site of greatest silica accumulation in cereals. Rice husks, for example, may contain up to 20% silica by dry weight. Engel (1953) suggested that there was a combination between silica and galactose in cell walls but I don't believe that this finding has been repeated in subsequent investigations of plant tissues.

Carlisle: Pectin is also high in silicon and polygalacturonic acid.

Volcani: Dr Masuyuki Katsumata in my laboratory (unpublished) developed a column chromatography system (Sephadex G-25 and G-20, eluting with water) for the separation of monomeric silicic acid and polymeric polysilicate, and a Dowex-1 borate column for isolating a polysilicate-binding compound. He isolated from the cells of the diatom *Navicula pelliculosa* a polysilicate-binding sulphated polysaccharide containing fucose, mannose and an unidentified sugar (4:1:1). A similar polysaccharide was also isolated from the cell wall of this diatom by Dr Nilima Banerji (unpublished).

Perry: The plant system we have studied consists of single cell hairs. The polysaccharides associated with the silica are the usual polysaccharides of plant cell walls: cellulose, arabinoxylan, mixed linkage $\beta(1\rightarrow3)(1\rightarrow4)$ glucan, mannan and only trace quantities of uronic acids.

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General discussion

Williams: So far we have been dealing with mineral solutions. In this general discussion we should next deal with any remaining questions about biominerals before we move on to how silicon affects humans. I have a question about the relationship between the mineral world and the biomineral world. What percentage of the soil, to a depth of about 30 cm, has been through a biological system?

Farmer: According to Wilding et al (1977) the amounts of opal phytoliths in soils commonly range from less than 0.1% to 3% of the total soil, but the amounts can be much higher. For example, the surface horizon of an East African soil under acacia, bamboo and fern consisted almost completely of opal phytoliths.

Werner: How stable are these phytoliths?

Farmer: The lifetime of opal phytoliths in soil will depend on the plant species, the soil type and the climate. Bartoli & Souchier (1978) report a mean lifetime for beech phytoliths of only three years in a brown soil but 27 years in a podzol. The lower solubility of the phytoliths in the podzol may be due to adsorption of aluminium, which is known to lower the solubility of silica gel. Phytoliths of beech and grasses are smaller and more soluble than those of conifers. Bartoli & Souchier (1978) report mean lifetimes of 80 years for fir and 300 years for pine in podzols, compared with 27 years for beech.

Williams: If the amount of silica in grasses is so high, one should be able to calculate roughly how much tonnage of silica goes through the grasses on the surface of the earth per year and multiply that by 10^9 (for the age of the cooled earth) to get the total SiO_2 turnover. An enormous amount of silica must go through the biosphere.

Farmer: You could calculate this from the data I presented for podzols in Table 1 of my paper (p 12). In such acid soils, with little or no earthworm activity, phytoliths will remain with the leaf debris in the upper humus layer. The concentration of silicon in solutions draining the humus layers (0.6–3.4 mg/l), must be largely derived from the dissolution of phytoliths, and so represent biocycled silicon. Silicon in solutions draining from the lower B horizon (2.2–3.6 mg/l) must arise from mineral weathering, when a steady state has been established. Bartoli (1983) concluded that 85% of the soluble silicon in soil was derived from biogenic silica under a deciduous forest, but only 15% under a coniferous forest.

Birchall: I have read somewhere that in soil growing barley there is 3% of opaline silica by mass.

Williams: All we need to know now is the stability or half-life of phytoliths in a field of barley.

Sangster: Previously, the question was raised as to the mechanism by which some plants, notably dicotyledons, exclude silica. Biochemical information on this subject is lacking. There is speculation that a suberin-like substance in cell walls of the root hairs, where much uptake occurs, might depress the influx of silicic acid into the roots of low Si-accumulator plants.

Williams: We are dealing with the dead system first so I want to make sure that everybody feels satisfied that the relationship of the mineral world to the biological minerals has been established to the best of our ability.

Sullivan: Isn't the question of biological cycling over geological time more relevant than the question of the turnover rate between soil and plants? The dissolution of biogenic silica and mineral silica in crustal rock and its biological precipitation into oceans to create oceanic sediments is a bigger picture.

Dobbie: A few years ago we made ^{31}Si. It might be appropriate to check ^{32}Si.

Volcani: ^{32}Si is not useful for biochemical studies because of its transmutation to ^{32}P, and it is extremely difficult to prepare. ^{31}Si is much more accessible. Unfortunately it has a very short half-life of only 156 minutes, and it is too expensive to work with.

Dobbie: I wondered whether ^{32}Si would help in investigating silicon distribution in the biomass.

Werner: Another question is whether there is a different way for template-oriented mineralization to occur which leads to a highly ornamented structure versus a non-template-oriented mineralization and deposition. I would avoid the term inorganic and say that in your diagram (Fig. 1, p 2) we have to draw a line to each of these two processes.

Williams: I am trying to say that the mineral deposited is as dead as dust, even though it has been through biology. It is finished with biology and is just lying around in the earth. Later the question will come up as to whether the hazards of dusts of various kinds are only mineral or are both mineral and biomineral.

Now I want to focus on something rather different in the life process. We are nearly always confounded by the biological system because it puts energy and messages into the ordinary inorganic processes of silica. Therefore we have to go all the way back to DNA to be able to understand what we are talking about in the life process. People constantly say there is a transport mechanism and that it can be switched on, that there is a 'pre former' and that can be switched on, and that it can be damaged by damaged tubule systems and so on. In other words, much of what we visualize inside biology is programmed all the way back to the DNA. In Dr Volcani's work that is well demonstrated for diatoms. In almost every other form of life it has not been demonstrated that any cellular communication goes back to the DNA but we begin to suspect it does because

of all the different modes of accumulation and deposition in different species. The patterns are species-specific and cell-specific. It all looks as if it is one of those fantastic worlds of chemistry which is under biological control. Perhaps the way silica is handled in every organism we talk about goes back to the DNA.

Last: What are being nominated for templates are glycans and xylans. The current dogma is that those are synthesized stochastically rather than being encoded by RNA and DNA. So if indeed we are templating on polysaccharides there is a problem, because this can be a random stochastic process rather than each molecule having a precisely defined structure.

Williams: Some of the molecules are glycosylated proteins in these systems. As well as poly sugars, proteinaceous material is associated with the silica.

Last: Then we have to come up with templates other than those described today.

Williams: Perhaps that is true. We have to take the individual systems.

Volcani: We are making things more complex rather than simpler. We are basically concerned with two sets of reactions. One is what we talked about here: what do we know, what don't we know, and what would we like to know? Evidently the transport system in the diatom as well as in the plant is still not understood and much work has still to be done. Secondly, is there a matrix and if so what is it? Then we want to know what processes are entailed in the accumulation of silicate as well as in the polymerization of silica. This is really what is missing here.

Then we are moving towards silicon at the molecular level: what do we know about that? We have been concerned with that for some time in our laboratory. From our extensive studies on the requirements of silicon for the syntheses of DNA and the nuclear DNA polymerases, we know definitely that silicon is involved in gene expression. The evidence is as strong as one can have for the concept of gene expression. We are trying to find how it operates. Using recombinant DNA we are isolating silicon-dependent genes and have discovered a number of plasmids which would, one hopes, serve well as vectors for transformation and so on. We also know that silicon is essential for cyclic AMP synthesis and the regulation of cyclic AMP, and that cyclic AMP may be coupled to the synthesis of DNA. We can now pursue that at the molecular level.

From Edith Carlisle's work we know that silicon affects the syntheses of collagen and glycosaminoglycan polysaccharides in bone formation. We don't know what those processes are.

Why don't we know anything about silicon in other systems? There are two reasons. First, the quantities of silicon required for these processes are minuscule. Second, while silicon is a blessing to our planet, it is an enigma to the biochemist because no system is free of it. Every chemical is contaminated with it, therefore we can only study the question of the DNA in a system which has been demonstrated to be controlled tightly by silicon. The diatom provides

such a system and we can really show what the consequences are at a specific stage in the cell cycle.

We tried to do this with the mammalian system and we failed because there is already too much silicon in the media in which we grow cells. A simple experiment might answer whether *E. coli* require silicon, if only we had a medium that is absolutely free of silicon. But is it possible to get a silicon-free medium? The answer is most probably 'not right now'. However, if we had a compound with the capacity to sequester silicate we might be able to sequester it in the medium, and show what the consequences are on the growth of the organisms. This would help us to understand the role of silicon in life processes. Elucidation of the structure of the silicate ionophore-like compound that we found may give us the clue for the synthesis of the silicate-sequestering compounds.

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A primer on organosilicon chemistry

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Abstract. Organosilicon chemistry is easily understood because a few generalizations allow us to compare it with the chemistry of carbon, hydrogen and the metals. A silyl group is used in organic synthesis as a metallic substitute for a hydrogen atom, metallic enough in character to control both the site of attack by an electrophile and the position of the final double bond. The silyl group can be thought of as a kind of super-proton. Organosilicon biochemistry is beginning to exist because of the possibility of incorporating silyl groups into drugs, and because the secondary metabolism of silicon-containing compounds is being studied.

1986 Silicon biochemistry. Wiley, Chichester (Ciba Foundation Symposium 121) p 112–122

The possibility of a silicon-based life analogous to carbon-based life is a seductive idea to science-fiction writers, but there is little chance that it exists anywhere in the universe. The chemistry of silicon is far less rich than that of carbon, although there is still an immense amount of published work on silicon chemistry. My purpose in this paper is to summarize very briefly the salient features (Fleming 1979, Colvin 1981, Weber 1983) of only part of that work—organosilicon chemistry, which is the chemistry of silicon compounds having one or more bonds to carbon.

The most striking difference between carbon and silicon is that:

Double bonds to silicon are very weak

Whereas carbon has a very rich double-bond chemistry (indeed *most* of organic chemistry is π-chemistry), the chemistry of silicon is almost all concerned with single bonds to silicon. There is a very limited π-chemistry of silicon: the challenge to force double bonds on a reluctant silicon, including the pioneer work of F. S. Kipping in the early years of this century, has stimulated much organosilicon chemistry, but only recently, and after much ingenious research, have stable compounds with Si=C and Si=Si double bonds been made. There is, therefore, little prospect that a silicon-based life system can ever develop the wide range of reactions found in organic chemistry, so many of which are necessary for the biochemistry we know. Nevertheless, silicon does have a substantial chemistry, and I want now to indicate what that chemistry is,

emphasizing inevitably its effect on organic chemical reactions in molecules in which a silyl group is built into the structure.

Fundamental silicon chemistry
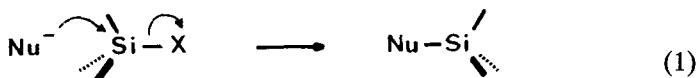
The main feature of silicon chemistry is that:

The bonds from silicon to electronegative elements such as oxygen and the halogens are very strong

This is what we see all around us in the silica and silicate structures, which have already had time to reach a low position on the thermodynamic scale. Nevertheless, in spite of their strength:

The bonds from silicon to the electronegative elements are easily broken and re-formed

The reaction in which they are made and broken is the most common event in silicon chemistry, the $S_N2(Si)$ reaction (Equation 1). This reaction-type allows the silica and silicate lattices to be broken up and re-formed. It is, for example,

$$Nu^- \quad Si-X \longrightarrow Nu-Si \tag{1}$$

the type of reaction taking place when glass is dissolved by hydrofluoric acid, for fluoride ion is a powerful nucleophile towards silicon (in contrast to its low nucleophilicity towards carbon). The chemistry of silicon is characterized, therefore, by the *thermodynamic strength* of silicon–heteroatom bonds, and by their *kinetic weakness*. These characteristics are typical of metals, and silicon is in almost every chemical sense a metal, although a feeble one in most respects. Most significantly, it is more electropositive than carbon, and therefore:

The silicon–carbon bond is polarized in the sense Si→ C
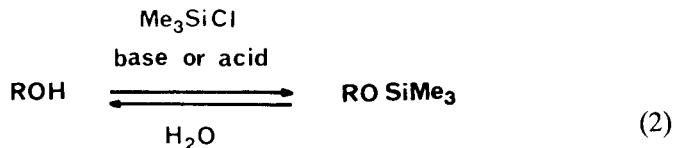
Organosilicon chemistry

There are, as far as I know, no silicon–carbon bonds in nature, but compounds with silicon–carbon bonds are now readily available because of the silicone industry. The monomer of the silicone industry is made by the copper-catalysed reaction of methyl chloride with silicon, which gives the monomer Me_2SiCl_2 as the major product along with a large number of by-products. The simple polymer is made by the hydrolysis of this compound, which does not lead to the silicon equivalent of acetone because of the weakness of the silicon–oxygen double bond. Varying amounts of $MeSiCl_3$ and Me_3SiCl (and other compounds) are used to cross-link or to terminate the growing chains. This is a large-scale industry, and all these compounds, including the by-products,
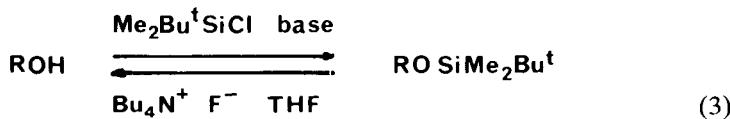
are relatively cheap. Their availability has stimulated organic chemists to think of them as reagents in organic synthesis, and I want now to say just a little on this subject, since it is my own.

Organosilicon chemistry in organic synthesis
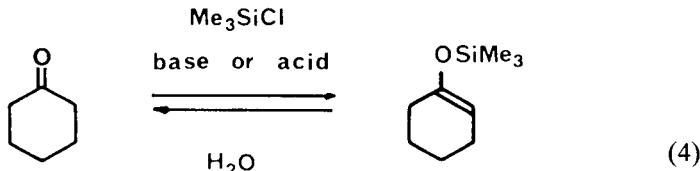
The most common use of silicon compounds in organic chemistry is the protection of OH groups by silylation (Equation 2), which is a special case of Equation 1. The silyl ethers are much less polar than the corresponding

$$\text{ROH} \underset{\text{H}_2\text{O}}{\overset{\substack{\text{Me}_3\text{SiCl} \\ \text{base or acid}}}{\rightleftharpoons}} \text{RO SiMe}_3 \tag{2}$$

alcohols and, because they do not engage in hydrogen bonding, are much more volatile than their hydroxylic precursors (Pierce 1968). This makes them more suitable for physical techniques such as distillation, gas chromatography and mass spectrometry. For example, even sugars such as glucose can be distilled when they are silylated on all the OH groups. These ethers are easily hydrolysed, in fact rather too easily for organic synthesis, so more stable silyl ethers are usually used when a protecting group is needed. The most commonly used group is the t-butyldimethylsilyl, which can be put on under fairly mild conditions and removed rather selectively with fluoride ion (Equation 3), but is

$$\text{ROH} \underset{\text{Bu}_4\text{N}^+ \ \text{F}^- \ \text{THF}}{\overset{\text{Me}_2\text{Bu}^t\text{SiCl} \ \text{base}}{\rightleftharpoons}} \text{RO SiMe}_2\text{Bu}^t \tag{3}$$
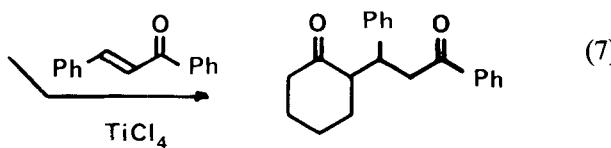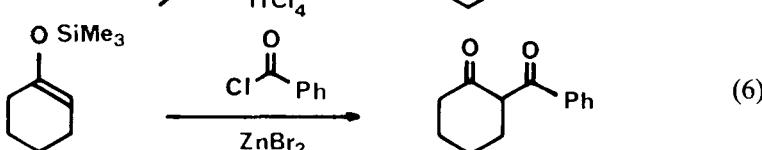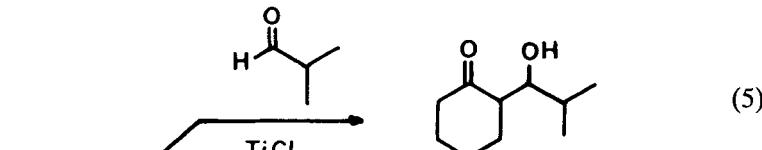
stable to many reaction conditions used by organic chemists. A special kind of silyl ether is the enol silyl ether prepared, using Lewis acid or base and trimethylsilyl chloride, from aldehydes, ketones or esters (Equation 4) (Brownbridge 1983). These compounds have the reactivity of enols, but they can be

$$\tag{4}$$

isolated and purified, which enols cannot. They then react with a wide variety of electrophiles, duplicating in their overall chemistry much of what has

traditionally been achieved, with much less control, using enols and enolate ions. Thus there is now a silyl enol ether version of all the traditional enolate ion reactions, such as the aldol (Equation 5), Claisen (Equation 6), Michael

$$(5)$$

$$(6)$$

$$(7)$$

(Equation 7), Mannich, Reformatsky, and alkylation reactions taught in all introductory courses in organic chemistry.

There is an even richer, although more specialized, chemistry of carbon-bound silicon. The silicon–carbon bond is usually made by the attack of nucleophilic carbon on electrophilic silicon (Equation 8), but it can also be made using nucleophilic silicon (in the form of a silyl–cuprate reagent) and

$$(8)$$

electrophilic carbon. Once made, the Si–C bond is fairly strong and resists breakage in the conditions of many organic reactions. To be broken, the carbon must be activated, and this is usually achieved by manipulating the functional groups to give a carbonium ion (or its operational equivalent, an alkyl halide) β to the silicon (Equation 9). In this situation, nucleophilic attack

$$(9)$$
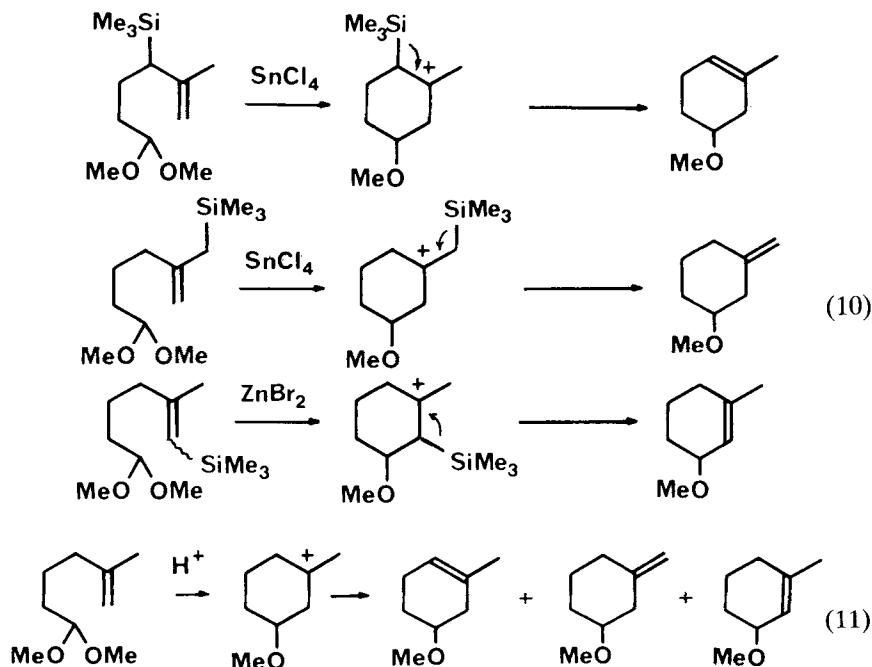
selectively removes the silyl group and gives a double bond. The striking features here are that:

An intermediate β silyl cation is thermodynamically stabilized by the neighbouring Si–C bond
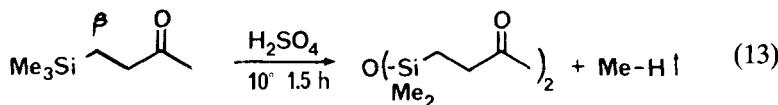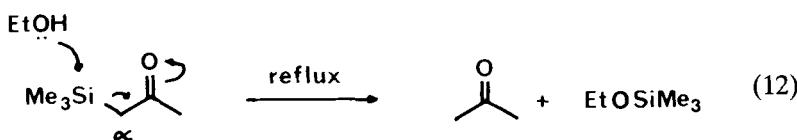
and that the silyl group is just metallic enough so that:

The silyl group is lost slightly more easily than a proton
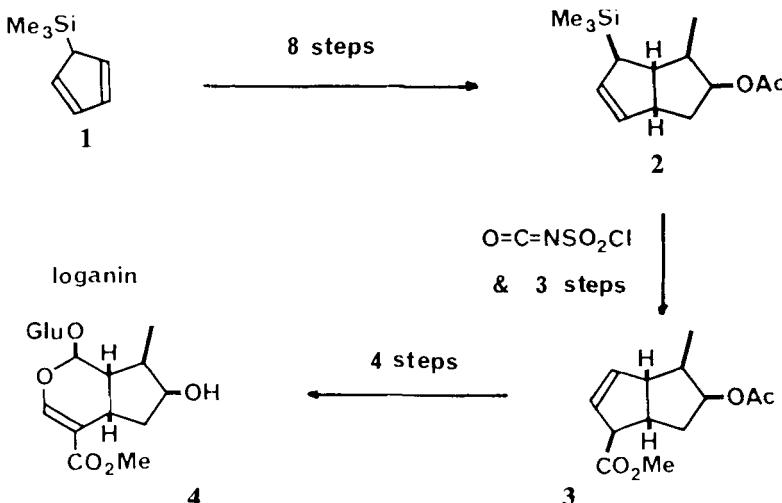
We can see most vividly how this allows us to control organic reactions if we contrast the outcome of the three reactions in Equation 10 (Chow & Fleming 1984) with the uncontrolled reaction in Equation 11. A silyl group is therefore a powerful element for the control of the position of a double bond in organic

synthesis. However, it is not unique in this respect—there are methods for achieving this sort of control based on phosphorus and sulphur, to name only the most commonly used elements. What makes silicon unique is the very feebleness of its metallic properties. If we go back to the last generalization above, the comparison of silicon with hydrogen tells us that we can predict when silicon will be easily removed from carbon—if a proton can be lost from carbon, silicon will be lost a little more easily, but if a proton is not easily removed, then a silyl group will be very firmly bonded to the carbon. This is illustrated by the contrast in Equations 12 and 13: where the silicon is α to the carbonyl group it is lost easily, just as protons easily come off from this position in the enolization process, but with silicon β to the carbonyl it is firmly bonded,

$$\text{(12)}$$

$$\text{(13)}$$
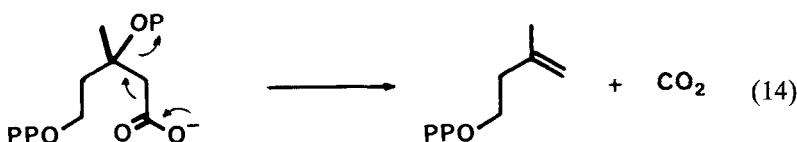
and only a methyl group comes off even in strongly acidic conditions. This knowledge makes for much security in synthetic planning, as I can show in our loganin synthesis (Au-Yeung & Fleming 1981). The silyl group was easily introduced (Equation 8), then survived eight steps (**1**→**2**) in which functional-

8 steps

1

2

$O=C=NSO_2Cl$

& 3 steps

loganin

4 steps

4

3

ity was developed before being used in the key step (**2**→**3**), where it does its duty by controlling both the site of attack by the chlorosulphonyl isocyanate and the position of the double bond in the product. The remaining steps (**3**→**4**) are conventional organic chemistry, but they rely on the double bond being where the silyl group placed it. The planning of this synthesis was greatly helped by our confidence that the silyl group in the intermediates between **1** and **2** would be stable to all the reaction conditions, because a hydrogen in this position could be relied upon to survive. In all this chemistry, the by-product when the silyl group is lost from the organic fragment is trimethylsilanol or a trimethylsilyl halide. These compounds invariably give hexamethyldisiloxane, $(Me_3Si)_2O$, on work-up, and this molecule is conveniently volatile (b.p. 101°C) and simply disappears into the atmosphere.

Perhaps I can make a comparison here with a biochemical process in which

$$\text{(scheme)} \qquad + \quad CO_2 \qquad (14)$$
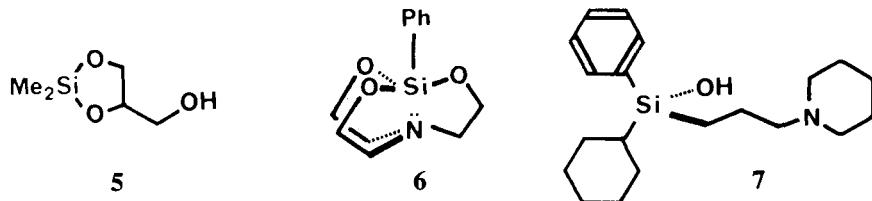
the outcome of a carbonium ion reaction is controlled. The decarboxylative elimination giving isopentenyl pyrophosphate (Equation 14) has a carboxylate ion as the electrofugal group controlling the position of the double bond. The carboxylate ion is nature's silyl group.

Bio-organosilicon chemistry
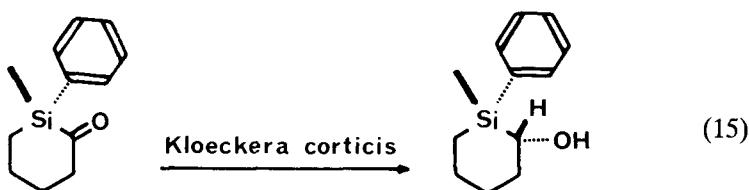
Since there are no Si–C bonds in nature, bio-organosilicon chemistry is a highly artificial subject, but it is just beginning to exist.

In the first place, people have naturally thought of incorporating silyl groups into potential drugs, perfumes, and the like (Tacke & Wannagat 1979). I know only of RDN (5) (Crowe 1983), which is said to be used in France against arteriosclerosis, but there are claims for several substances in the Russian reports, such as the silatranes (6) (Voronkov 1979), many of which are toxic. The obvious first step for making silicon-containing drugs is to replace a carbon

5   **6**   **7**

or phosphorus atom in a known drug by a silicon atom. For example, the antimuscarinic derivative R-sila-procyclidine (7) resembles the tertiary carbinol procyclidine, but is more acidic and of higher potency (Tacke 1985). This sort of compound is called a sila-drug. Sila-drugs, so far, appear to resemble the parent drug in their pharmacology, but it should be easy to adjust the shape and size of hydrophobic regions of a drug molecule by trying silyl groups of various sizes, and the long Si–C bond should make it possible to change a cyclic framework into something of different shape and size without necessarily changing the polarity much.

A second kind of bio-organosilicon chemistry is the secondary metabolism of silicon-containing compounds (Fessenden & Fessenden 1980). Organosilicon compounds are highly artificial substrates, but the less selective enzymes can be expected to accept them, as in the diastereoselective reduction of the acylsilane in Equation 15. As organosilicon compounds become more widespread,

$$\xrightarrow{\text{Kloeckera corticis}} \qquad (15)$$

organosilicon toxicology must grow. At the moment, tetraorganosilicon compounds enjoy widespread use in organic chemistry without anyone knowing of any serious danger associated with them. It seems unlikely that this situation will last indefinitely.

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DISCUSSION

Volcani: Can you comment on the likelihood of finding silicon–carbon bonds in nature?

Fleming: Silicon carbide exists, but I don't imagine that any organisms could start with this and try to keep the silicon–carbon bond. The standard way of making a silicon–carbon bond is to reflux a Grignard reagent with a silyl halide. Those kinds of conditions would have to be duplicated: nature would have to increase the nucleophilicity of a carbon species, to make it powerful enough to react with silica, or raise the electrophilicity of silica, in order to make it as

electrophilic as a silyl halide. That's a pretty tall order. Yet nature is able to make a cobalt–carbon bond. Nature might be able to use a cobalt–carbide bond as the nucleophile, but it's not as nucleophilic as a Grignard reagent. I doubt if you could take the coenzyme of vitamin B_{12} and a silyl halide and make a carbon–silicon bond.

Werner: With cobalt you already have a cation available, but with silicon there is only silica, and it is the Si–O bond which you have to split first.

Fleming: You have to activate the Si–O bond, although Grignard reagents will displace the alkoxide from silyl ethers; it is a slower reaction than with the silyl halide. I suppose one could make a better leaving group, perhaps a phosphate, on the silicon and some means of ensuring that the carbon of the nucleophilic species attacked the silicon and not the phosphate. I am sure that, given time, nature might find a solution to this problem.

Williams: Carbon is transferred (as the methyl group) from B_{12} to tin, platinum, mercury, arsenic, or lead. Whether there is a silicon compound to which the carbon could be transferred, I don't know.

Sullivan: Germanium which behaves similarly to silicon has been methylated by biological process in the marine environment. Dr P. Froelich's group has been studying the distribution of germanium in estuarine and oceanic systems (Andreae & Froelich 1984). They have found both monomethyl germanium and dimethyl germanium at levels of about 300 pM and 100 pM in sea water. It seems to be produced biologically from sediments, possibly by bacterial action.

Volcani: Germanium reacts in two ways, as a metal, a cation, and as an ion, whereas silicon does not react in that dual way.

Fleming: It seems that we are edging towards finding carbon–silicon bonds, and it may well be only a matter of time before they are found.

Mann: Can you suggest any chemical mechanisms for stabilizing the silicon–catechol type of complex, by perhaps changing residual groups around the phenol ring?

Fleming: The traditional organic chemistry solution would be to put two large groups on silicon, because that would greatly increase the ease of ring formation. You could use di-*t*-butyldichlorosilane.

Williams: It seems to me that in order to protect silicon from general hydrolysis one probably has to have carbon bound to it somewhere. What is interesting is that the Si–O–Si bond itself doesn't seem to be open to attack, once three methyl groups are also attached to the Si. You can break the bond of course, chemically, but in normal biological conditions, in water, it would be expected to remain, as it does in silicone chemistry. It slightly surprises me that it doesn't hydrolyse.

Fleming: Perhaps the disilyl ether is simply a thermodynamically stable arrangement. Do we know that it is not hydrolysing and re-forming all the time?

Williams: I wondered about this, and whether there is exchange. I suspect

that the oxygen does not exchange. This chemistry is intriguing, because it is a way in which one could maintain a structure with silicon, while it is moved around in a biological system. I don't myself think that carbon–silicon bonds will be found in biological systems generally, but I think that an odd methylation may be possible here and there.

Another problem has intrigued me. If you make silicon compounds which have four carbon bonds to the Si, then you have a silicon atom pretty well tied up and nothing much ever happens to it. Biological processes cannot attack it. This protection offers enormous potential in the drug industry. Putting Si in place of a carbon atom in a carbon framework alters the distances between every functional group by a very small amount, about 0.01 or 0.02 nm. So all one needs to do is look for quaternary carbons in any particular compound, put silicon in place of carbon, and see what can be done with the new compound in a biological setting. After all, the way to manipulate chemistry against biology is not to make dramatic changes, because then the biological specificity is deficient.

Fleming: I agree. Silicon will be perfect for tailoring a molecule to fit a receptor; when you know exactly what you want, you can introduce different elements, especially silicon because of its stability, to tailor precisely the shape of the group that you want to fit.

Williams: I am surprised that the drug industry is not already doing that, now we know so much about the shapes and sizes of enzymes. Obvious places to start will be in steroid chemistry or wherever else there are quaternary carbons.

Fleming: Of course, until 1968, organic chemists didn't think much about silicon at all. Then the organic chemistry of silicon began to develop dramatically, partly because cheap reagents became available, and partly because a few key synthetic chemists made the subject popular. I would be surprised if drug companies by now are not doing this kind of thing.

Williams: Another approach which is common in carbon chemistry and in drug development is to transfer a methyl group, using a methylating agent. Examples are alkylating agents of the mustard gas type, which are anticancer agents. An extension of this principle to other elements is possible. The *cis*-platinum drugs, for example, seem to be based on platinum resembling carbon and attacking DNA. Silicon is much nearer carbon, so we might be able to develop a good silicon reagent, working like a carbonium centre, but using different protecting substituents. One could not use chloride because it is too rapidly hydrolysed. What is a good slow leaving group from silicon?

Fleming: A silyl ether would have to be used, or something similar like a hydride. Is it known whether the alkylations or platinations take place on oxygen or nitrogen?

Williams: It was originally thought that many of the anti-cancer alkylating agents attacked DNA at the nitrogen bases, but that is not the only reaction since they also attack at the oxygen centres of the sugar of DNA. So if

silicon-type chemistry could be devised such that Si attacked the sugars of DNA and not the bases, it would give us a new set of alkylating drugs which would prefer oxygen centres.

Fleming: The problem is to make the silylating agent stick on the sugar and not hydrolyse off.

Williams: Once you have an Si–O–C bond, the question is the one I asked earlier: how stable is it? The stability clearly depends on what one chooses for substituent groups.

Fleming: The problem is that you will have to introduce silicon bonded to ether oxygen; if you bring it in on anything else, it will come off in the hydrolytic medium, before it reaches the intended site of action. Any kind of silyl carboxylate, for instance, hydrolyses as soon as it is put in water. You might be able to design a silyl group that is slowly enough hydrolysed, but then the sugar will find it just as difficult to displace the silyl group.

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Silicon as an essential trace element in animal nutrition

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Abstract. Within the last decade silicon has been recognized as participating in the normal metabolism of higher animals and as being an essential trace element. Silicon is found to perform an important role in connective tissue, especially in bone and cartilage. Bone and cartilage abnormalities are associated with a reduction in matrix components, resulting in the establishment of a requirement for silicon in collagen and glycosaminoglycan formation. Silicon's primary effect in bone and cartilage is on the matrix, with formation of the organic matrix appearing to be more severely affected by silicon deficiency than the mineralization process. Additional support for silicon's metabolic role in connective tissue is provided by the finding that silicon is a major ion of osteogenic cells and is present in especially high concentrations in the metabolically active state of the cell; furthermore, silicon reaches relatively high levels in the mitochondria of these cells. Further studies also indicate that silicon participates in the biochemistry of the subcellular enzyme-containing structures. Silicon also forms important inter-relationships with other elements. Although it is clear from the body of recent work that silicon performs a specific metabolic function, a structural role has also been proposed for it in connective tissue. A relationship established between silicon and ageing probably relates to glycosaminoglycan changes.

1986 Silicon biochemistry. Wiley, Chichester (Ciba Foundation Symposium 121), p 123–139

Within the last decade, silicon has been recognized as an essential trace element participating in the normal metabolism of higher animals. We have shown that silicon is required in bone, cartilage and connective tissue formation as well as participating in several other important metabolic processes. Although interest in the silicon content of animal tissues and the effect of siliceous substances on animals was expressed over half a century ago (King & Belt 1938), emphasis has been placed until recently on the toxicity of silicon, its effect on forage digestibility, on urolithiasis and especially on silicosis (caused by dust inhalation). This paper is mainly concerned with information that has extended the physiological significance of silicon in nutrition. This results in emphasis being placed on silicon's role in connective tissue metabolism.

Essentiality

A series of experiments has contributed to the establishment of silicon as an essential element. The first experiments were *in vitro* studies in which we showed that silicon is localized in active growth areas in bones of young mice and rats, suggesting a physiological role of silicon in bone calcification processes. These were followed by *in vivo* studies showing that silicon affects the rate of bone mineralization. Of critical importance, we subsequently demonstrated (Fig. 1) that silicon deficiency is incompatible with normal growth and skeletal development in the chick and that these abnormalities could be corrected by a silicon supplement (Carlisle 1972). During the same year Schwarz & Milne showed that silicon deficiency in the rat results in depressed growth and skull deformations. Later studies, both *in vitro* and *in vivo*, emphasize silicon's importance in bone formation and connective tissue metabolism and confirm the postulate that silicon is involved in an early stage of bone formation. Some of these studies are discussed in this presentation.
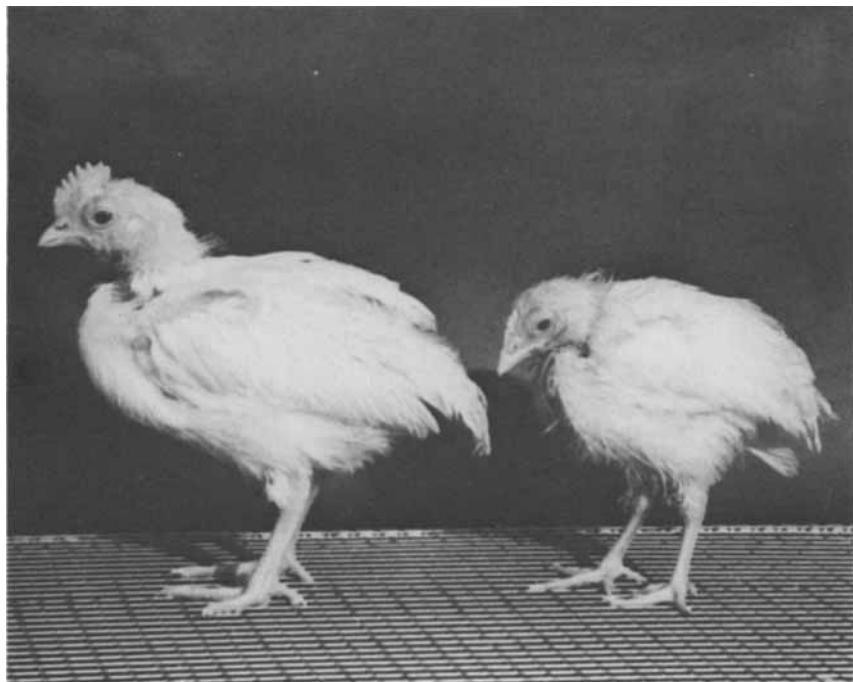
FIG. 1. Four-week-old chicks on silicon-supplemented diet (left) and low-silicon basal diet (right) (Carlisle 1972).

Tissue silicon

Earlier findings on the silicon content of living tissues varied greatly, and, in general, reported values were considerably higher before the advent of plastic laboratory ware and the development of suitable methods. Even with more recent methods, considerable variance still exists in reported tissue concentrations of silicon (Schwarz 1978).

Normal human serum has a narrow range of silicon concentration, averaging 50 µg/dl (Carlisle 1986a); the range is similar to that found for most of the other well-recognized trace elements in human nutrition. The silicon is present almost entirely as free soluble monosilicic acid. No correlations of age, sex, occupation or pulmonary condition with blood silicon concentrations have been found, although the level increased when silicon compounds were specifically administered.
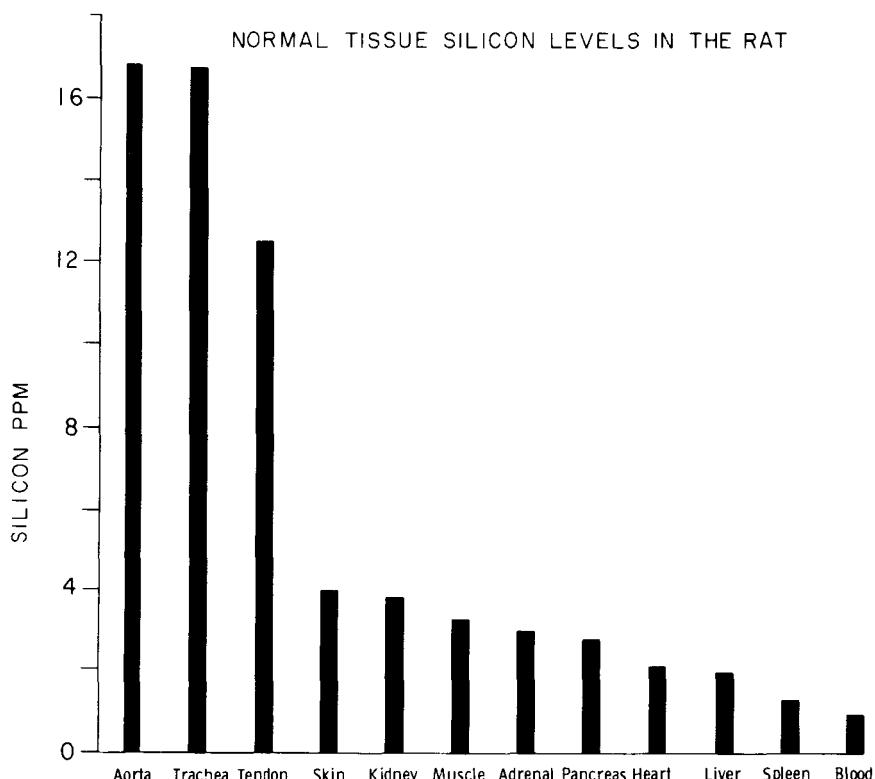
FIG. 2. Normal tissue silicon levels in the adult male rat. Values represent mean silicon levels in 20 animals (4 months of age) expressed as parts per million wet weight of tissue. (From Carlisle 1974.)

Connective tissues such as aorta, trachea, tendon, bone and skin and its appendages are unusually rich in silicon, as shown by studies of several animal species (Carlisle 1974). In the rat, for example (Fig. 2), the aorta, trachea and tendon are four to five times richer in silicon than liver, heart and muscle. The high silicon content of connective tissues appears to arise mainly from its presence as an integral component of the glycosaminoglycans and their protein complexes which contribute to the structural framework of this tissue. Fractionation procedures reveal that connective tissues such as bone, cartilage and skin yield complexes of high silicon content. Silicon is also found as a component of glycosaminoglycans isolated from these complexes.

The consistently low concentrations of silica in most organs do not appear to vary appreciably during life. Parenchymal tissues such as heart and muscle, for example, range from 2 to 10 µg of silicon/g dry weight (Carlisle 1986). The lungs are an exception. Similar levels of silicon have been reported in rat and rhesus monkey tissues, where soft tissue levels in both species varied from 1 to 33 µg of silicon/g dry weight, except in the primate lung and lymph nodes, which averaged 942 p.p.m. and 101 p.p.m. respectively. High levels in human lymph nodes have been associated with the presence of clusters and grains of quartz (Carlisle 1986a).

Silicon deficiency and function

Calcification

The first indications of a physiological role for silicon were from this laboratory, reporting that silicon is involved in an early stage of bone calcification. In electron microprobe studies (Carlisle 1970) silicon was shown to be uniquely localized in active growth areas in young bone of mice and rats (Fig. 3). The amount present in specific very small regions within the active growth areas appeared to be uniquely related to 'maturity' of the bone mineral. In the earliest stages of calcification in these regions the silicon and calcium contents of the osteoid tissues were both found to be very low, but as mineralization progressed the silicon and calcium contents rose congruently. In a more advanced stage the amount of silicon fell markedly, such that as calcium approached the proportion present in bone apatite, the silicon was present only at the detection limit. In other words, the more 'mature' the bone mineral, the smaller the amount of measurable silicon. Further studies of the Ca:P ratio in silicon-rich regions gave values below 1.0 compared with a Ca:P ratio of 1.67 in mature bone apatite. These findings suggested strongly that silicon is involved in an organic phase during the series of events leading to calcification.

Subsequent *in vivo* experiments showed that silicon has a demonstrable effect on *in vivo* calcification (Carlisle 1974); that is, a relationship between the level of dietary silicon and bone mineralization was established. Weanling rats
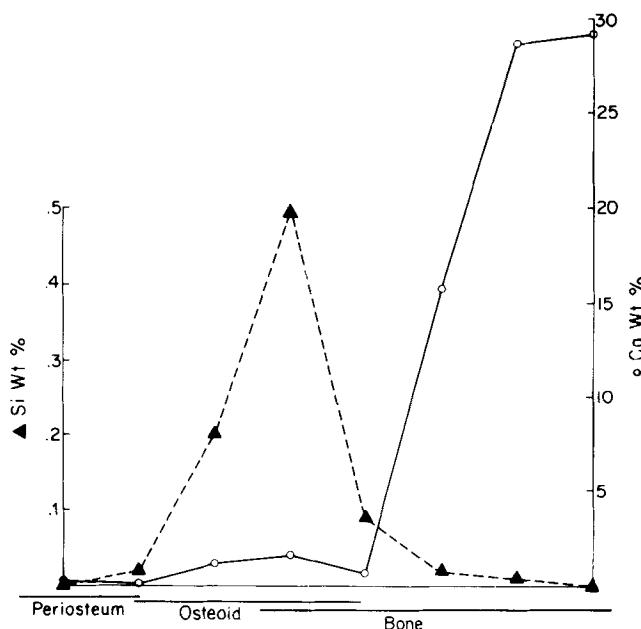
FIG. 3. A spatial relation between silicon (▲) and calcium (○) composition (% by weight) in a typical traverse across the periosteal region of young rat tibia (cross-section) using microprobe techniques. (Reproduced from Carlisle 1970 with permission.)

were maintained on diets containing three levels of calcium (0.08, 0.40, 1.20%) at three levels of silicon (10, 25, 250 p.p.m.). An increase in silicon in the low calcium diet resulted in a highly significant (35%) increase in the percentage of ash in the tibia during the first three weeks of the experiment. Silicon was found to hasten the rate of bone mineralization. The calcium content of the bone also increased with increased dietary silicon, substantiating the theory of a relationship between mineralization and silicon intake. The tendency of silicon to accelerate mineralization was also demonstrated by its effect on bone maturity, as indicated by the [Ca]:[P] ratio. The concept of an agent that affects the speed of chemical maturity of bone is not new. Muller et al (1966) found that the chemical maturity of vitamin D-deficient bone, although inferior to control bone during the period of maximum growth, approaches the control level at the end of the experiment.

Bone formation

The earliest studies suggesting a role for silicon in bone formation were those mentioned above. Most significant, however, was the establishment of a silicon

deficiency state incompatible with growth and normal skeletal development. In the chick, this is evidenced by the reduced circumference, thinner cortex and reduced flexibility of the leg bones as well as by smaller and abnormally shaped skulls, with the cranial bones appearing flatter (Carlisle 1972). Silicon deficiency in rats was also shown to result in skull deformations (Schwarz & Milne 1972).

Recent studies further emphasize the importance of silicon in bone formation. Skull abnormalities associated with reduced collagen content have been produced in silicon-deficient chicks under conditions promoting optimal growth, on a diet containing a natural protein in place of the crystalline amino acids used in earlier studies (Carlisle 1980a). An additional finding was the striking difference in the appearance of the skull matrix between the silicon-deficient and silicon-supplemented chicks: the matrix of the deficient chicks totally lacked the normal striated trabecular pattern of the control chicks. The deficient chicks showed a nodular pattern of bone arrangement, indicative of a primitive type of bone.

Using the same conditions, and introducing three different levels of vitamin D, I showed that the effect exerted by silicon on bone formation is substantially independent of the action of vitamin D (Carlisle 1981a). All chicks on silicon-deficient diets, regardless of the level of dietary vitamin D, had gross abnormalities of skull architecture; furthermore, the silicon-deficient skulls showed considerably less collagen at each vitamin D level. As in the previous study, the bone matrix of the silicon-deficient chicks totally lacked the normal striated trabecular pattern of the control chicks. In the rachitic groups of chicks, the appearance of the bone matrix was quite different from that in the groups receiving adequate vitamin D, being considerably less calcified and more transparent, so enabling the cells and underlying structure to be seen more easily. The deficient chicks appeared to have markedly fewer osteoblasts than the controls. In these two studies, the major effect of silicon appears to be on the collagen content of the connective tissue matrix and this is independent of vitamin D.

Cartilage and connective tissue formation

In addition to its effect on bone, silicon deficiency is manifested by abnormalities involving articular cartilage and connective tissue (Carlisle 1976). Chicks in the silicon-deficient group had thinner legs and smaller combs in proportion to their size. Long-bone tibial joints were markedly smaller and contained less articular cartilage than those of silicon-supplemented chicks. The deficient chicks also revealed a significantly lower hexosamine content in their articular cartilage (Table 1). In cock's comb also, a smaller amount of connective tissue, a lower total percentage of hexosamines and a lower silicon content were found in the silicon-deficient group. These findings point clearly to an involvement of
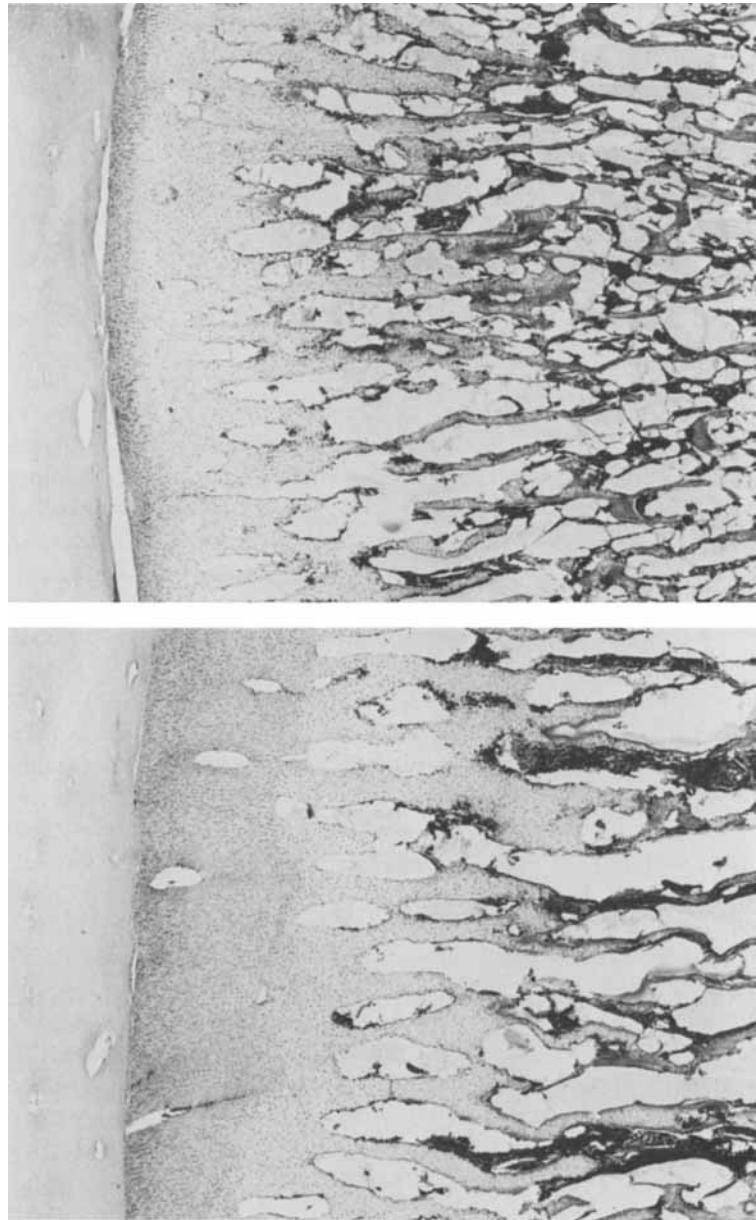
FIG. 4. Longitudinal section through the proximal end of the tibia from 4-week-old chicks fed a silicon-supplemented diet (left) and low-silicon diet (right). In the silicon-deficient chick note the great reduction in width of the epiphyseal cartilage lying below the cartilaginous epiphyses, especially striking in the narrow zone of proliferating cartilage cells. The proliferative zone of silicon-deficient chicks is seven to eight times narrower than that of silicon-supplemented chicks. Also note the pale-staining matrix of the hypertrophic zone underlying the proliferative zone and the absence of widely, uniformly spaced tunnelling. Haematoxylin–eosin stain × 40. (From Carlisle 1980b.)

TABLE 1   Effect of silicon intake on articular cartilage composition[a]

Diet	Tissue (mg wet wt)	Total hexosamine (mg wet wt)	Percentage hexosamine (% wet wt)
Low silicon	63.32 ± 8.04	0.187 ± 0.23	0.296 ± 0.009[b]
Supplemented	86.41 ± 4.82	0.310 ± 0.031	0.359 ± 0.011

[a] Twelve chicks per group. All values reported as mean ± SD.
[b] Significantly different from the supplemented animals at $P < 0.001$.

silicon in glycosaminoglycan formation in cartilage and connective tissue.

In more recent studies, long-bone abnormalities similar to those reported above have been produced in silicon-deficient chicks given a diet containing a natural protein in place of the crystalline amino acids used in the earlier studies (Carlisle 1980b). Tibia from silicon-deficient chicks had significantly less glycosaminoglycan and collagen, the difference being greater for glycosaminoglycans than collagen. Tibia from silicon-deficient chicks also showed rather marked histological changes, profound changes being demonstrated in epiphyseal cartilage (Fig. 4). The disturbed epiphyseal cartilage sequences resulted in defective endochondral bone growth, indicating that silicon is involved in a metabolic chain of events required for normal growth of bone.

Connective tissue matrix

The preceding *in vivo* studies have shown silicon to be involved in both collagen and glycosaminoglycan formation. Silicon's primary effect in bone and cartilage appears to be on formation of the matrix, although silicon may also participate in the mineralization process itself. The *in vivo* findings have been corroborated and extended by studies of bone and cartilage in organ and cell culture.

Studies in which embryonic skull bones were grown in culture (Table 2) further demonstrate the dependence of bone growth on the presence of silicon (Carlisle & Alpenfels 1978). Most of the increase in growth appears to be due to a rise in collagen content; silicon-supplemented bones showed a 100% increase in collagen content over silicon-low bones after 12 days. Silicon is also shown to be required for formation of glycosaminoglycans; at day 8, the increase in hexosamine content of supplemented bones was nearly 200% more than in silicon-low bones, but by day 12 it was the same in both groups.

A parallel effect has been demonstrated in the growth of cartilage in culture and is especially marked in cartilage from 14-day embryos compared with 10-day and 12-day embryos (Carlisle & Alpenfels 1980). Silicon's effect on collagen formation was also especially striking in cartilage from 14-day

TABLE 2   Effect of silicon on rate of synthesis of bone matrix components

Days in culture	Bone chondroitin sulphate[a]		Bone collagen[b]		Bone non-collagenous protein[c]	
	Silicon		Silicon		Silicon	
	Low	Suppl.	Low	Suppl.	Low	Suppl.
4	0.51	7.42*	0	62.7*	241	236
8	3.58	10.50*	64.2	117.9*	158	102
12	6.14	5.90	89.5	176.1*	200	188

[a] Hexose nitrogen × 2.56.
[b] Hydroxyproline = 7.46.
[c] Leucine = NH_2 nitrogen corrected for collagen and hexose nitrogen.
* Significantly different from the supplemented media at $P < 0.05$.

embryos (Fig. 5), appearing to parallel the rate of growth. Similarly, matrix hexosamines (glycosaminoglycans) were formed more rapidly by silicon-supplemented cartilage, the most striking difference in this case being in cartilage from 12-day embryos. The requirement for silicon in collagen and glycosaminoglycan formation thus proves not to be limited to bone matrix but to apply also to cartilage.

An interaction between silicon and ascorbate (Carlisle & Suchil 1983) has also been shown in cartilage. Silicon's effect on cartilage formation was investigated in the presence and absence of ascorbate. No significant effect on hexosamine content occurred in the absence of ascorbate. However, silicon supplementation resulted in significant increases in wet weight, hexosamine and proline content in the presence of ascorbate. The effect on hexosamine content was greater than that on proline. Furthermore, silicon and ascorbate interact to give maximal production of hexosamines. Silicon also appears to increase hydroxyproline, total protein and non-collagenous protein independently of the effects of ascorbate.

An effect of silicon on the formation of extracellular cartilage matrix components by connective tissue cells has also been demonstrated (Carlisle & Garvey 1982) in chondrocytes isolated from chick epiphyses cultured under silicon-low and silicon-supplemented conditions. The major effect of silicon appeared to be on collagen. Silicon-supplemented cultures demonstrated a 243% ($P<0.01$) increase in collagen measured as hydroxyproline over low-silicon cultures. Silicon also had a pronounced stimulatory effect on matrix polysaccharides; the matrix polysaccharide content of silicon-supplemented cultures increased 152% ($P<0.01$) more than that of low-silicon cultures. Silicon's effect on collagen and glycosaminoglycan formation was not due to cellular proliferation but to some system in the cell participating in their formation.
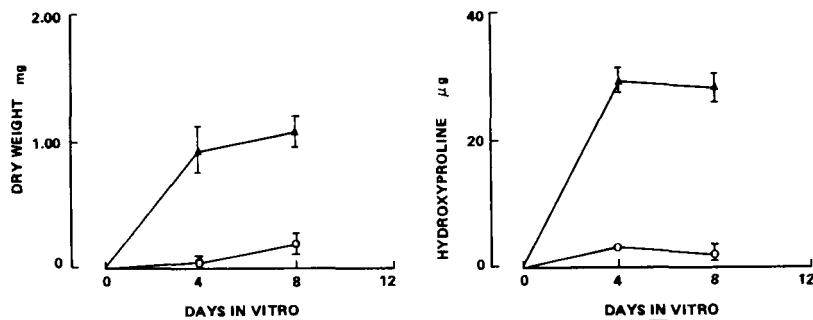
FIG. 5. Rate of growth (left) and collagen synthesis (right), measured as hydroxyproline, in chick tibial epiphyseal cartilage from 14-day-old embryos in culture (Carlisle & Alpenfels 1980). ○, low Si; ▲, Si-supplemented.

We have also shown that maximal prolyl hydroxylase activity depends on silicon (Carlisle et al 1981). Prolyl hydroxylase obtained from the frontal bones of 14-day-old chick embryos incubated for four or eight days under low silicon conditions with 0, 0.2, 0.5, or 2.0 mM-Si added to the media showed lower activity in low-silicon bones, with increasing activity in 0.2, 0.5 and 2.0 mM cultures. The results support the *in vivo* and *in vitro* findings of a requirement for silicon in collagen biosynthesis, the activity of prolyl hydroxylase being a measure of the rates of collagen biosynthesis.

Recent studies suggest a mitochondrial role for silicon in the synthesis of proline precursors (Carlisle & Alpenfels 1984). Studies in which epiphyseal cartilage from 12-day and 14-day embryos was grown in culture were continued on a larger scale and proline was determined in addition to analyses for hexosamine, hydroxyproline and non-collagenous protein as previously. In 12-day cultures by far the most obvious difference was with proline synthesis; large differences between deficient and silicon-supplemented media at days 4 and 8 suggest the possibility of a role for silicon in the proline synthetic pathway.

Additional support for silicon's metabolic role in connective tissue at the cellular level is provided by evidence of its presence in connective tissue cells (Carlisle 1982). X-ray microanalysis of active growth areas in young bone and isolated osteoblasts show silicon to be a major ion of osteogenic cells, the amounts of silicon being in the same range as that of calcium, phosphorus and magnesium. Moreover, silicon appeared to be especially high in the metabolically active state of the cell, the osteoblast. Clear evidence that silicon occurs in the osteoblast and is localized in the mitochondria adds strong support to the proposition that silicon is required for connective tissue matrix formation.

Structural component

Although the discussion above indicates that silicon plays an important metabolic role in connective tissue, a structural role has also been proposed, mainly supported by the finding that in connective tissue silicon is a component of animal glycosaminoglycans and their protein complexes. In higher animals, the glycosaminoglycans—hyaluronic acids, chondroitin sulphates and keratan sulphate—are found to be linked covalently to proteins as components of the extracellular amorphous ground substance that surrounds the collagen, elastic fibres and cells. By extraction and purification of several connective tissues, we have shown silicon to be chemically combined in the glycosaminoglycan fraction. The silicon content of the glycosaminoglycan–protein complex extracted in this laboratory from bovine nasal septum, for example, is 87 p.p.m. compared to 13 p.p.m. in the original dried cartilaginous tissue (Carlisle 1976). From this complex, smaller molecules considerably richer in silicon were isolated. Silicon was found to be associated with the larger, purer polysaccharide and smaller protein moieties.

Similar results on isolated glycosaminoglycans, which included some reference research standards, have been reported by Schwarz (1973). More recently, however, Schwarz has reported (Schwarz 1978) that many of his earlier observations on the occurrence of bound silicon in glycosaminoglycans were in error because they were based partially on results obtained with materials contaminated by silica or polysilicic acid. Work in our laboratory shows that silicon is indeed a component of the glycosaminoglycan–protein complex; however, the amount of silicon in these complexes is less than the values reported by Schwarz (1973) for isolated glycosaminoglycans.

The preceding results indicate that silicon is not merely involved in glycosaminoglycan formation but that, in animal glycosaminoglycans at least, and quite probably in plant polysaccharides, it is also a structural component.

Interaction with other elements

An interrelationship between silicon and molybdenum has recently been established (Carlisle 1979). Plasma silicon levels were strongly and inversely affected by molybdenum intake; silicon-supplemented chicks on a liver-based diet (Mo 3 p.p.m.) had a plasma silicon level 348% lower than chicks on a casein diet (Mo 1 p.p.m.). Molybdenum supplementation also reduced silicon levels in those tissues examined. Conversely, plasma molybdenum levels are also markedly and inversely affected by the inorganic silicon intake. Silicon also reduced molybdenum retention in tissues. The interaction occurs within normal dietary levels of these elements. Although a copper–molybdenum–sulphate interrelationship has been shown in animal species, this is the first work demonstrating a silicon–molybdenum interaction.

Aluminium is another element with which silicon is shown to form an interrelationship. Since the establishment of silicon as an essential trace element (Carlisle 1972), all tissues analysed for silicon have been analysed simultaneously for aluminium and a number of other elements. From the many analyses of tissues we have done in several animal species (E.M. Carlisle, unpublished work 1985) we have established a relationship between silicon and aluminium which may have relevance for Alzheimer's disease in humans (Carlisle 1986b). Other papers in this symposium deal with the silicon–aluminium interrelationship (Birchall 1986, this volume, and Edwardson 1986, this volume).

Ageing

Because connective tissue changes are prominent in ageing, it is not surprising to find a relationship between silicon and ageing in certain tissues. The silicon content of the aorta, other arterial vessels and skin was found to decline with age, in contrast with other analysed tissues, which showed little or no change (Carlisle 1974). The decline in silicon content was significant and was particularly dramatic in the aorta, commencing at an early age. This relationship was seen in several animal species.

In human beings, the silicon content of the skin dermis has been reported to diminish with age. In contrast with an earlier finding, French investigators (Loeper et al 1978) reported that the silicon content of the normal human aorta decreases considerably with age; furthermore, the level of silicon in the arterial wall decreases with the development of atherosclerosis. The potential involvement of silicon in atherosclerosis has been suggested by others (Schwarz et al 1978, Dawson et al 1978). It is of possible significance here that a relationship has been reported between silicon, age and endocrine balance, and it is suggested that the decline in hormonal activity may be responsible for the changes in silicon levels in senescence (Charnot & Peres 1971).

In contrast to the decrease in silicon content with age found in certain connective tissues, the accumulation of silicon in certain other tissues, mainly due to environmental influences, raises the possibility that a failure to dispose of silicon may also affect the ageing process. In humans, it was shown in an earlier study (King & Belt 1938) that silicon levels gradually increase with age in the human peribronchial lymph nodes, even in subjects who have no history of unusual exposure to dust. More recently, in Alzheimer's disease (Nikaido et al 1972), a presenile condition characterized pathologically by the presence of glial plaques in the brain, an unexpectedly high increase in silicon has been reported in the cores and rims of the senile plaques. The precise relationship of silicon with the ageing process remains to be determined.

Conclusion

Silicon is one of the most recent trace elements in nutrition to be established as 'essential' for higher animals, and a mechanism and site of action have been identified. Silicon has been demonstrated to perform an important role in connective tissue, especially in bone and cartilage. It is clear from the body of recent work that silicon performs a specific metabolic function. However, a structural role has also been proposed for silicon in connective tissue. A relationship has been established between silicon and ageing which is probably related to glycosaminoglycan changes. The precise relationship of silicon with the ageing process remains to be determined.

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DISCUSSION

Daniele: Have you looked at collagen metabolism in fibroblasts?

Carlisle: No, we had planned to do this using 3T3 cells. I suspect that it is similar to what we have found in chondroblasts.

Richards: We looked at the effects of mineral dusts including silica on lung fibroblasts (Richards & Hunt 1983). The results are a little different from yours. Growth (as measured by time-lapse cinematography [Absher & Sylwester 1981] or by the DNA content of the culture) is promoted in the same way as in chondroblasts, but when collagen (hydroxyproline) deposition is expressed in terms of DNA, control and quartz-treated cultures are much the same. So there seems to be an initial growth-promoting effect of silica and then a later extra deposition of collagen and increased glycosaminoglycan metabolism. I was therefore surprised that in your chick silicon deficiency studies *in vivo* there were enormous differences between chick tibia in the numbers of chondroblasts, yet when your data are expressed in terms of DNA, one almost expects some of your collagen synthetic rates to go down, rather than up, after silicon supplementation.

Carlisle: Fig. 4 showed that the supplemented chick tibia did have a considerably greater number of chondroblasts in the proliferative zone of the articular cartilage. Analyses showed that the shaft which includes the epiphyseal cartilage at both ends did not contain significantly more collagen but did have a significantly greater amount of glycosaminoglycans. However, in the *in vitro* study when chondrocytes from embryonic chick cartilaginous epiphyses were grown in culture there was considerably more collagen and glycosaminoglycans, both total and expressed per milligram of DNA.

Richards: Which cell is making the collagen?

Carlisle: Chondroblasts in the growth plate and also osteoblasts in other parts of the tibia.

Henrotte: We have also studied the effect of silicon on cellular growth, looking at human peripheral blood lymphocytes in culture, using monomethylsilanetriol salicylate at a concentration of 5 mg silicon per litre. The salicylate was present to stabilize the molecule and prevent its polymerization. The incorporation of [^{3}H]thymidine gave us an estimate of cell proliferation. Our preliminary results show a significant enhancement of cell proliferation by the silicon compound (J.G. Henrotte and S. Claverie-Benureau, unpublished results). When a known mitogen, either concanavalin A (Con A) or pokeweed mitogen, was added to the cell culture, silicon had no additional effect on [^{3}H]thymidine incorporation. We obtained the same kind of results when salicylate was replaced by glucose. These results suggest that silicon and the mitogen compete for the same receptor on the cell surface. Since the receptors for Con A and pokeweed mitogen are glycopeptides, this observation would fit with the known properties of silicon in binding to glycans.

Carlisle: We measured DNA content along with the matrix components in our chondroblast culture studies and found that at higher silicon concentrations there was an inhibitory effect of silicon on DNA synthesis.

Richards: I think it is true that you reach a toxic level, where the cells are killed. At a level of silicon just below that you may stimulate DNA synthesis, or growth; at a lower level still, you may affect collagen or glycosaminoglycan metabolism (Richards & Curtis 1984).

Henrotte: In our experiments on lymphocytes, if we use a silicon concentration 10 times greater (50 mg/l rather than 5 mg/l), there is no stimulatory effect on cell proliferation. In some cases, there is even an inhibitory effect. At these higher concentrations, we may reach a toxic level.

Williams: Are you suggesting that in silicon deficiency, proline is a very susceptible synthesis?

Carlisle: Yes; silicon appears to be involved in the synthesis of proline necessary for the synthesis of deoxycollagen, supplying more substrate for the hydroxylation reaction.

Williams: If silicon is involved in proline synthesis, one would expect it to affect a large number of proteins, because there is a range of proline-rich proteins. Several proteins in saliva, for instance, are very rich in proline. So one

would expect them to be affected by silicon and the ratio of proline to all other amino acids in the total protein should be reduced.

Carlisle: I haven't checked that, but the non-collagenous protein is also affected by lack of silicon.

Williams: It needs a reference point, to establish that it is an effect on proline rather than something to do with collagen, because the total proline content would apparently go down if only collagen synthesis failed at any stage.

Carlisle: When 'deoxycollagen' is formed, proline is needed for that. Also, for hydroxylation, you need α-ketoglutarate, a proline precursor. So one is affecting collagen synthesis at two different places.

Williams: Then you are almost into the Krebs cycle, and that affects all amino acids; so the question is whether you have a baseline for saying that proline itself is affected rather than that the cells have to produce a lot of proline because they have to produce collagen. This might simply be indicative of a general synthetic problem, not just of proline.

Hench: Do you see any effects of silicon on matrix vesicles that would correlate with these extracellular effects?

Carlisle: We have just finished isolating matrix vesicles and are now performing the chemical analyses. Just previous to this we fractionated epiphyseal cartilage from growing chicks into microsomes, lysosomes and mitochondria. We found very high silicon levels in microsomes, and in an enriched lysosomal fraction.

Sullivan: Do you have any model or working hypothesis for the molecular action of silicon in any of these processes? Is silicon acting as a small-molecule effector, or is it covalently bonded or hydrogen-bonded in some way that is influencing a number of processes, as you have shown?

Carlisle: Unfortunately, I cannot supply a satisfactory answer at present. However, I think that in bone formation, for example, silicon is acting as a small-molecule effector.

Hench: Have you looked at the effects of silicon on dentition in the rat model?

Carlisle: Dr K. Schwarz has noticed an effect of silicon on pigmentation in the rat, but other elements, such as fluoride and tin, had the same effects, so it wasn't specific. I haven't noticed any gross effects of silicon on dentition in the silicon-deficient rat, but I have not looked at the microscopic level.

Werner: You discussed the interaction between molybdenum and silicon. These molecules are rather different. Molybdate biochemistry is linked to a number of enzymes—nitrate reductase, xanthine oxidase and nitrogenase, for instance. What exactly is the point of interaction of these two trace elements?

Carlisle: Molybdenum appears to reduce the absorption of silicon from the diet, resulting in reduced plasma silicon levels and a reduction in tissue levels, as mentioned in my paper. Nearly all the colorimetric methods for measuring silicon in tissues are modifications of the silicon–molybdate method, indicating that they must react very readily.

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Biological implications of the interaction (via silanol groups) of silicon with metal ions

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Abstract. Since the demonstration, in 1972, of the essentiality of silicon in higher animals, bio-inorganic chemists have speculated about the site and mechanism of action of silicon. Bone and connective tissue have been identified as tissues that are altered in the absence of silicon. Si–C bonds are foreign to biochemistry so organic bonding must be via Si–O–C, but the instability of the ester bond in aqueous solution at pH 7.4 has prompted us to investigate the interactions of silicic acid. Silicic acid could, by hydrogen bonding, alter the conformation of organic macromolecules, since hydrogen bond association can inhibit silanol condensation. However, silanols are also able to interact with metal ions that are basic at physiological pH, e.g. Fe^{3+} or Al^{3+} but not Ca^{2+}, and such interactions are known in geochemistry.

The observed effects of Si on hard and soft tissue could therefore result from interactions of $Si(OH)_4$ with Fe^{3+}, which is involved in connective tissue synthesis (via enzymes, e.g. prolyl hydroxylase) and damage (via iron-catalysed radical generation), or with Al^{3+} which exerts toxic effects at sites (bone and brain) at which Si has also been observed. Although we have demonstrated several Fe^{3+}/Si interactions, we have not been able to show their relevance in a biochemical context. Al^{3+} interacts with $Si(OH)_4$ in aqueous solution and preliminary experiments have suggested that silicic acid can counteract deleterious effects of aluminium, for example the activity of prolyl hydroxylase, an observation with implications not only in osteogenesis but also in Alzheimer's disease and aluminium toxicity in acidified waters.

Silicon is essential in the diatom, not only for the formation of the frustule, but also because it appears to be involved in major metabolic processes. Schwarz & Milne (1972) and Carlisle (1972) independently showed a dietary requirement for silicon in rats and chicks, the experiments indicating bone and connective tissue as sites altered in silicon deficiency. The element was claimed to be bound to the polysaccharide and protein components of connective tissue, the claimed levels falling as analytical techniques improved. In subse-

quent studies Carlisle (1984) has found silicon within the osteoid layer of growing bone and within the osteoblast, the evidence suggesting effects on the formation of the pre-osseous matrix and on mineralization.

There is much speculation about the mechanism and exact site of action of silicon (Birchall 1978) in higher animals. Has silicon, as has often been proposed, a structural role in connective tissue biopolymers—akin to sulphur in vulcanized rubber, which alters physical properties at 1 atom per 10 000 daltons—or a more subtle metabolic function?

There is no evidence for Si–C bonds in biology, so organic bonding must involve either Si–O–C bonds or hydrogen bonds via silanol groups. We have not succeeded in identifying *stable* Si–O–C species in aqueous solution at pH 7.4, although complexes exist at high pH values with 1,2–dihydroxyphenols. Hydrogen-bonded complexes between silicic acid and, for example, hydroxy compounds can be sufficiently stable to inhibit polycondensation, and the secondary structure of polymers could be influenced by such bonding. Indeed there is some evidence for such effects from studies of protein monolayers on water containing silicic acid (Minones et al 1984).

Certain hydrogen-bonded configurations can be uniquely stable. The silane triol, $CH_3O(CH_2CH_2O)_3(CH_2)_3Si(OH)_3$ is stable mainly as monomer in 50% aqueous solution (Birchall et al 1977). This stability is due to the formation of the 'tight' structure shown in Fig. 1 which screens the silanol groups and the constraint on the mobility of the polar chain is illustrated by ^{13}C lattice relaxation times, as shown in Table 1.

TABLE 1 ^{13}C spin lattice relaxation times measured on a polyether and polyether silane triol in 50% aqueous solution

CH_3O $CH_2CH_2OCH_2CH_2OCH_2CH_2O$	— CH_2 — CH = CH_2			
4.2 ⇐ 0.72 ⇒	1.9	5.1	2.0	(s)
0.72				
0.72				
1.10				
0.82				
1.13				

CH_3O CH_2CH_2O CH_2CH_2O CH_2CH_2O	— CH_2 — CH_2 — CH_2 — $Si(OH)_3$			
2.2 ⇐ 0.17 ⇒	0.10	0.08	–	(s)
0.19				
0.06				
0.06				
0.06				
0.40				
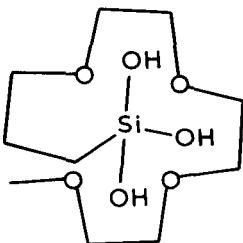
FIG. 1. Polyether silane triol—possible structure to give stability.

However, we have been able to demonstrate such dramatic effects only when Si is bound to the substrate via Si-C bonds, i.e. $(HO)_3$ Si–C. The apparent absence of such bonds in biology and the instability of Si–O–C bonds have led us to look at other chemical possibilities to explain the reported effects, particularly in osteogenesis. In particular we have considered the interaction of silicic acid and metal ions.

Interaction with metal ions

Silicic acid is a very weak acid ($pK_1 = 9.8$) that only interacts with metal ions at pH levels just below the level at which hydroxide precipitation occurs. This excludes Ca^{2+} and Mg^{2+} but suggests possible interactions at physiological pH with Fe^{3+} or Al^{3+}; these reactions are indeed of interest to water chemists and geochemists.

We were led first to a consideration of the reactions between Fe^{3+} and silicic acid at physiological pH, which have been little studied. The existence of Fe:Si 1:1 complexes has been established at low pH (<2) and at the ratio of 1:3 the precipitation of ferric hydroxide at pH 7 is said to be inhibited (Schenk & Weber 1968, Olson & O'Melia 1973). This is an embarrassment when water authorities want to remove Fe^{3+} from water supplies by filtering out the hydroxide.

An effect of $Si(OH)_4$ in 'solubilizing' Fe^{3+} would be of considerable biological interest in view of conjectures about the nature of the 'low molecular weight iron pool chelator' (Jacobs 1977), a role ascribed to several anions (citrate being favoured) but never to silicic acid, which is present in body fluids at a concentration similar to that of several other candidates. The iron pool is considered to be low in absolute concentration but of considerable kinetic importance in manipulating iron between transport and storage proteins and enzyme sites. Although experimentally intractable, this concept has found continued use in the explanation of many processes in iron metabolism.

A depletion of labile iron would result in the retardation of many iron-dependent reactions, including collagen synthesis. A recent suggestion has

been that silicon has a role in successful collagen synthesis related to the activity of prolyl-4-hydroxylase (EC 1.14.11.2: procollagen-proline, 2-oxoglutarate 4-dioxygenase) (Carlisle 1984).

The known co-factors of this important post-translational enzyme are O_2, Fe, ascorbate and α-ketoglutarate (Prokop et al 1976), of which only Fe has a known chemistry with silicic acid. Since the osteoblast produces collagen and contains Si, is the role of Si to aid the transport of essential iron, particularly since other ligands such as pyrophosphate and citrate can, *in vitro*, interfere with hydroxyapatite crystallization?

The 'mobilization' of Fe^{3+} by silicic acid

A 1:1 Fe^{3+} silicic acid complex is known at acidic pH but studies of complex formation at pH 7.4 are hindered by the extreme insolubility of iron in the absence of a complexing ligand. A threefold excess of silicic acid was sufficient to prevent the visible precipitation of ferric hydroxide (Table 2) although, predictably, the chelators desferrioxamine and nitrilotriacetic acid (NTA) both solubilized iron in accordance with their known 1:1 and 2:1 respective complexes. Citrate, on the other hand, inhibited precipitation at less than 1:1, suggesting polymerization of the Fe^{3+} species; this has been described by Spiro & Saltman (1969).

In view of these solubilizing effects we studied the ability of several Fe^{3+} complexes to donate iron to desferrioxamine. Fe^{3+} $(NTA)_2$ donated very rapidly whereas Fe^{3+}–EDTA, like iron oxide sol, gave a very low transfer rate. Both citrate and silicate gave intermediate donation rates, but with an important difference. The ability of the silicic acid complex to donate iron fell with age, whereas the citrate complex became more reactive with time, as shown in Fig. 2.

TABLE 2   Required ratio of [ligand] : $[Fe^{3+}]$ to prevent precipitation of $10^{-4}M$ Fe^{3+} at pH 7.4

<1:1	1:1	2:1	3:1	>10:1	>?
Catechol (0.5)[a]	EDTA	NTA	Silicate	Salicylate (40)[a]	Sucrose (>60)
					Glucose (>60)
Pyrophosphate (0.05–0.2)[a]	Desferri-oxamine			Methyl silane triol (50)[a]	Fructose (>60)
					Galactose (>60)
Citrate (0.05)[a]					
					Glycerol (>120)
					Orthophosphate (>660)

[a] Numbers in parentheses: actual ratio.
NTA, nitrilotriacetic acid.
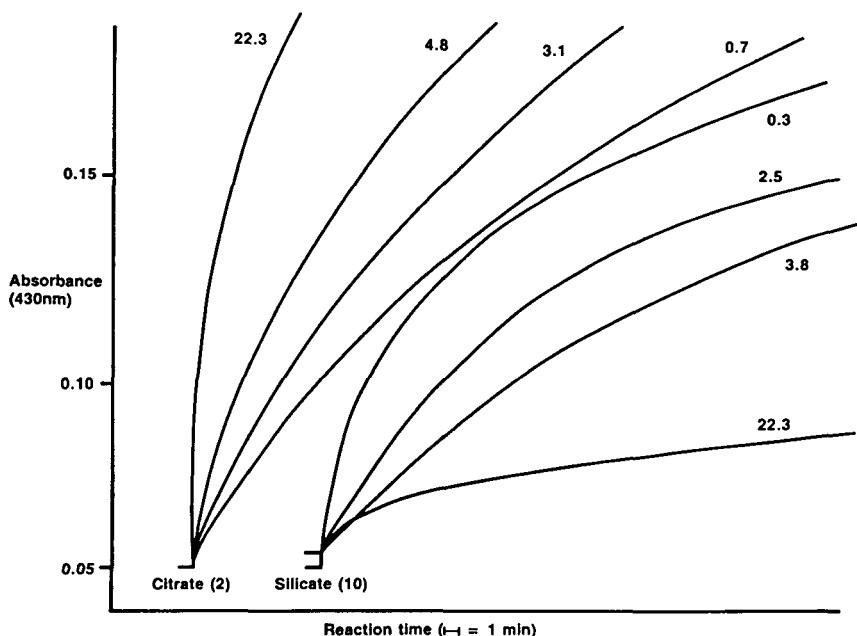
FIG. 2. Donation of chelated iron to desferrioxamine in bicarbonate buffer at pH 7.4, measured by absorbance of Fe-desferrioxamine complex. [Fe^{3+}], 10^{-4}; excess of ligand in brackets; number at each curve is ageing time in hours.

Such ageing effects may not be significant in the context of a transient intermediate carrier. Therefore we compared the ligands in terms of their ability to mediate the transfer of iron between a source complex, the transport protein transferrin, and desferrioxamine as the acceptor (Pollack et al 1976). A large increase in transfer rate was found for NTA and a smaller but significant increase for citrate, whereas silicic acid caused no significant increase.

The reasons for this different behaviour became clear after studies of the structure of citrate and silicic acid complexes by dialysis, gel filtration chromatography and electron microscopy. With both ligands at pH 7.4, Fe–O polymers are formed as colloidal particles. For silicic acid, these are particles of hydrated ferric oxide, 10–15 nm in diameter, stabilized against aggregation and precipitation by a coating of silicic acid. They are thus similar to ferrihydrite, an amorphous and poorly characterized mineral in soil chemistry (Schwertmann & Fechter 1982). With citrate, however, and in particular with an excess of ligand, species of low molecular mass exist alongside colloidal aggregates; indeed, the latter tend to depolymerize with time, thus aiding the supply of labile iron.

A reappraisal of a computational model for Fe^{3+} ligands in biofluids (P.M.
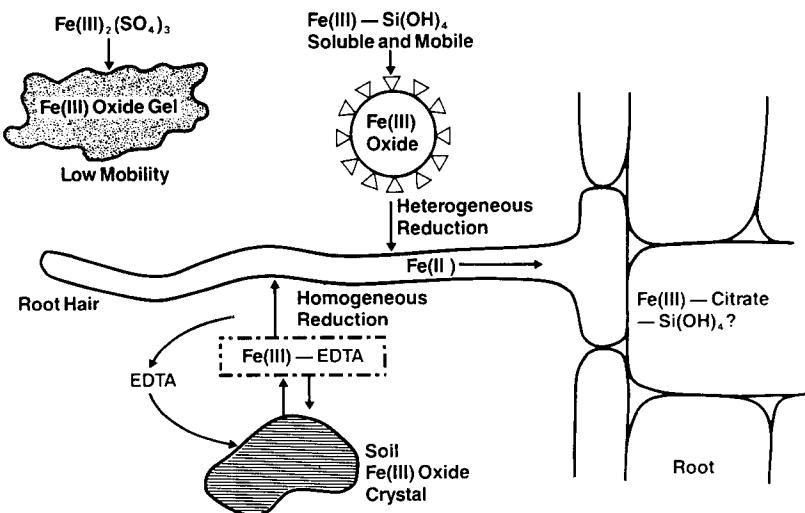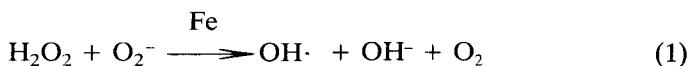
FIG.3. Iron uptake by plants.

May, personal communication 1984) has recently confirmed that complexation by silicic acid was insufficiently powerful to prevent hydrolysis or compete with other available ligands. Thus it seems doubtful that silicic acid can be regarded as a candidate chelator of labile iron pools in higher animals. However, our results explain the reported ability of silicic acid to assist iron uptake by plants (Demolon & Bastisse 1944). Ferric–EDTA complexes percolate easily to the plant root hair and are reduced by exuded reductants (Fig. 3). The addition of a common ferric salt (e.g. sulphate) to soil results in the immediate precipitation of iron hydroxide gel with little mobility. The application of Fe^{3+}–silicic acid solutions is an intermediate case, for the small (10 nm) particles of iron hydroxide formed are mobile and arrive at the root hair to be reduced, dissolved and absorbed, thus supplying essential iron to a chlorotic plant.

Reduction of the toxicity of iron deposits by silicic acid
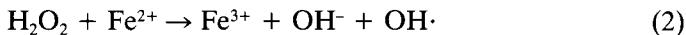
The adsorption of silicic acid onto the surface of Fe^{3+} hydroxides, exemplified by the soil mineral ferrihydrite and the analogous material described above, suggested to us a further possible effect of silicic acid in biology.

There is a great recent interest in reactions generating highly reactive species such as OH· (Halliwell & Gutteridge 1984), in which iron plays a central catalytic role, for example in the Haber–Weiss reaction:

$$H_2O_2 + O_2^- \xrightarrow{\text{Fe}} OH\cdot + OH^- + O_2 \qquad (1)$$

There is much debate about the ability of the various bound forms of iron (ferritin, transferrin, pool chelate) to engage in this reaction. Until recently little attention has been paid to the possibility of *heterogeneous* catalysis by 'deposits of iron hydroxide such as the poorly characterized haemosiderin, which occurs mainly in iron overload syndromes such as idiopathic haemochromatosis or secondary iron overload in thalassaemic patients, but also under 'normal' conditions, and is often associated with tissue damage, ceroid pigments, etc.

Although the iron in haemosiderin is Fe^{3+}, the resorption of the deposit most probably involves reduction and hence, in proximity to the surface, the reaction

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH\cdot \qquad (2)$$

We might therefore expect deposits of haemosiderin to be foci for local tissue damage and indeed this has recently been confirmed (Weir et al 1984).

Silicates are used commercially to reduce the rate of decomposition of peroxides and perchlorates, by the deactivation of adventitious particles of catalytic oxides (mainly iron). Similarly, iron oxide pigments in unsaturated polymers are treated with silicate to prevent the catalysis of oxidation. In soil chemistry, silicic acid can displace phosphate (at pH >7) from iron and aluminium oxides.

These related findings led us to suggest that silicic acid may act in a similar way in biology. Local micro-deposits of haemosiderin could result in local damage in, for example, arterial tissue or joints, and its deactivation could reduce this. Here we note the reported effect of silicic acid derivatives in reducing aortal fatty infiltration in cholesterol-fed rabbits (Loeper et al 1984) and the observation that in rabbits under such a regime the injection of iron–dextran causes iron and lipid to deposit together (Jankus 1973).

Iron oxide reactions affected by silicic acid

We have studied the effect of orthophosphate and silicic acid on the rate of decomposition of H_2O_2 by α–Fe_2O_3 sol. Typical results for silicic acid are shown in Fig. 4; orthophosphate results were very similar. Opposite effects are seen on either side of the isoelectric point for the sol. Silicic acid deactivated positively charged sols (pH <7) but increased activity at alkaline pH, when the surface charge is negative. The behaviour of silicic acid was found to depend on pH and the precise nature of the iron oxide species.

Studies of the ability of various ligands, including reductants, to dissolve ferric hydroxide showed pH dependence, with silicic acid having maximum activity at pH 8 (Fig. 5). Although silicic acid is less powerful than other agents
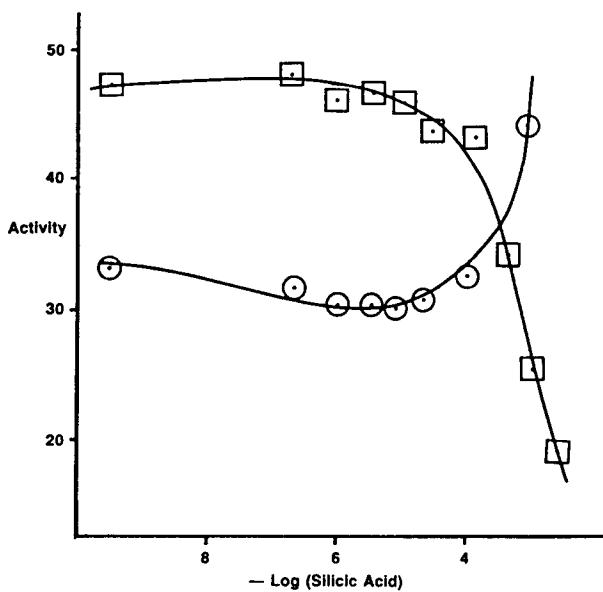
FIG. 4. Effect of silicic acid on H_2O_2 decomposition by α–Fe_2O_3 sol, at pH 5.6 ($\boxdot$) and 8.9 ($\odot$).
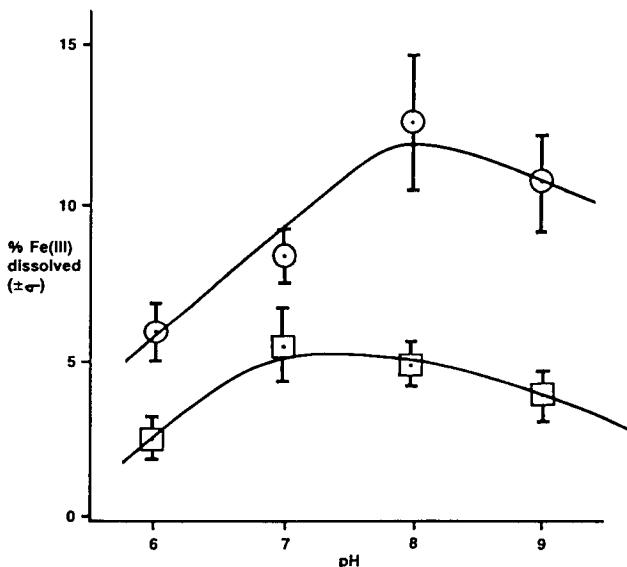
FIG. 5. Dissolution of iron oxide in bicarbonate buffer versus pH: the effect of silicic acid.
$[Fe^{3+}]_0$, $10^{-4}M$; reaction time, 2 hours; $[Si(OH)_4]$, 0 ($\boxdot$) and $7 \times 10^{-4}M$ ($\odot$).

such as citrate or desferrioxamine, its addition could increase the rate of Fe^{2+} production in the reductive dissolution of ferric oxide by ascorbate or lipid peroxidation induced by heterogeneous iron.

Thus silicic acid was found to dissolve or peptize iron oxide at physiological pH, with the soluble form being more reactive towards electron transfer processes than the insoluble form. Therefore we cannot ascribe to silicic acid a role in pacifying the surface of haemosiderin in a manner related to the industrial use of silicate to prevent undesirable catalysis.

Silicon aluminium chemistry and bone formation

The only reasonably well established effects of silicon in higher animals are related to connective tissue and osteogenesis. Silicon has been detected at active growth sites in young bone and is thought to be beneficial. It is curious to note that aluminium has also been observed at the bone mineralization front, resulting in osteomalacia—a condition characterized by thickened osteoid seams and impaired mineralization. Aluminium can enter the body in the course of long-term dialysis in the treatment of renal failure (De Broe & Van de Vyver 1985) and can also lead to severe and fatal dementia, somewhat similar to senile dementia of the Alzheimer type. Both silicon and aluminium have, independently, been found concentrated at sites of damage in Alzheimer's disease. These coincident occurrences of the two elements, particularly in bone, have led us to investigate possible chemical interactions in the context of bone formation and to question whether silicon can counteract a deleterious effect of aluminium. Aluminium could conceivably affect osteogenesis by influencing the synthesis of organic matrix or altering the growth of the mineral phase. Both these possibilities have been studied.

The aqueous chemistry of aluminium is, like that of iron, complicated. Both of these trivalent hydrated ions exist only at highly acidic pH. In less acidic solution, hydrolysis results in the formation of polynuclear species, which, for aluminium, are quite stable, e.g. $[Al_{13}O_4(OH)_{24}(H_2O)_{17}]^{7+}$. Depolymerization will be favoured by high dilution and complexation, and little is known about which species are present in the very low concentrations found in biological fluids and natural waters, although $Al(OH)^{2+}$ and $Al(OH)_2^+$ are likely. There is currently great interest in the mechanism of the toxicity of aluminium to fish in acidic waters.

Aluminium is similar in size and charge to ferric iron. It can be accommodated in transferrin and ferritin, and is removed from tissue in disease states by desferrioxamine. It has been shown to inhibit several enzymes, including Mn-dependent hexokinase (polysaccharide synthesis), the ferroxidase activity of caeruloplasmin, and the enzymic methylation of noradrenaline.

We have made a preliminary examination of the influence of aluminium on

TABLE 3   Effect of Al^{3+} and silicic acid on prolyl hydroxylase activity

Addition (100 μM)	% inhibition of control
$Si(OH)_4$	0
Zn	100
Fe then Al	20
Al then Fe	55
Al + 6 $Si(OH)_4$	0

Assays were done by measuring the release of ^3H as ^3H_2O from ^3H-prolyl protocollagen which accompanies the hydroxylation of proline at position 4.

A final volume of 0.5 ml was used for the reaction (5 ng purified prolyl-4-hydroxylase, 40μg ^3H-prolyl protocollagen, 100 μM-2-oxyglutarate, 1 mM-ascorbate, 50 μM Fe, 100 μM dithio-threitol, 0.1 mg mg^{-1} catalase, 2 mg ml^{-1} bovine serum albumin, all dissolved in 50 mM-tris-HCl buffer, pH 7.6).

The mixture was incubated at 37 °C and the reaction was stopped by adding trichloroacetic acid to a final concentration of 5% w/v together with ammonium sulphate (50% w/v saturation) and bovine serum albumin (10 mg). The precipitated protein was removed by centrifugation at 11 000 g for 10 min and a 1 ml sample of the supernatant was counted in a scintillation counter.

the activity of prolyl-4-hydroxylase in an assay of the purified enzyme (Peter-kovsky & Di Blasio 1975), with and without the addition of silicic acid (Table 3).

Our results for the addition of silicic acid alone to this assay confirm Carlisle's more recent suggestion (Carlisle 1984) that silicon is not considered an additional co-factor, but the activity of the enzyme is a measure of the rate of collagen biosynthesis, where Si is generally implicated. Zinc is known to inhibit by competitive binding with the iron co-factor. Aluminium is seen to be less powerful than zinc, but these early results, with the order of addition being important, suggest that aluminium can also deny the enzyme its essential redox co-factor. However, when aluminium is pre-mixed with silicic acid the inhibitory effect of aluminium is lost. The solution chemistry of aqueous silicon and aluminium at low concentration and pH 7 is ill understood, but the effect described above is likely to arise from the interaction of silicic acid with aluminium species. These reactions will involve the initial formation of soluble aluminosilicates from $Al(OH)^{2+}$, $Al(OH)_2^+$ and so on, and the eventual formation of colloidal solids, i.e. aluminosilicates of halloysite composition $(Al_2Si_2O_5(OH)_4)$ or the aluminium analogue of ferrihydrite.

Silicon–aluminium chemistry and mineralization

The mineral component of bone and teeth is hydroxyapatite, $(Ca_5(PO)_3OH)$,

the end-product of a complicated precipitation process that can be affected by factors such as supersaturation, organic substrates, accelerators, inhibitors, temperature and pH. While nothing appears to have been published on the effect of silicic acid on this reaction, the aluminium ion has been shown to inhibit the crystal growth of synthetic hydroxyapatite (Meyer & Thomas 1982). In tissue culture studies of embryonic chick bone by Miyahara et al (1984) aluminium was shown not to influence matrix formation or degradation, but to inhibit mineralization and stimulate demineralization.

It is probable that neither silicon nor aluminium can substitute into the hydroxyapatite lattice. For aluminium it is more probable that mixed Ca/Al phosphates prevent the nucleation, or form on the surface, of hydroxyapatite and retard growth. For silicon, a calcium silicate nucleating agent has been proposed by Iler (1979), but at pH <9 such species are not predicted.

We have studied the effects of aluminium, silicic acid and known inhibitors, e.g. pyrophosphate, on the precipitation and ageing of calcium phosphates. Our results are summarized in Table 4. The addition of silicic acid was shown not to alter the phase composition (or the microstructure, as seen by electron microscopy) when compared with the control reaction.

Both pyrophosphate and aluminium, however, had a similar and dramatic effect on this precipitation, with the $CaHPO_4.2H_2O$ being replaced by gelatinous non-crystalline material. In both cases this probably results from the rapid preferential formation of calcium pyrophosphate and aluminium phosphate respectively, which affects the normal progress of crystalline phase development.

Preliminary results have also been obtained on the effect of aluminium on the nucleation of hydroxyapatite in dilute solution at pH 7.4 (Boskey & Posner 1976). Under these conditions, in a supersaturation range claimed to be similar to that existing *in vivo*, hydroxyapatite can be formed directly, without an amorphous precursor. Analytical electron microscopy and diffraction were used to determine the phase and composition of the very small (5–100 nm) solid particles formed by this method. Particles with hydroxyapatite diffraction spacings and a Ca/P ratio of ~1.6 were formed in the control reaction ([Ca] 1.8 mM, [PO_4] 1 mM). However with the addition of 10^{-5} M–Al, no hydroxyapatite was produced and the amorphous products were composed of a mixture of phosphates containing both Al and Ca.

Our result for aluminium, where the nucleation of hydroxyapatite is prevented, adds to the previous results on the inhibition of *growth* of hydroxyapatite by Meyer & Thomas (1982) and suggests that Al may seriously interfere with the mineral component of bone deposition.

So far we have been able to show that, unlike Al, $Si(OH)_4$ does not adversely affect the initial precipitation of calcium phosphate. Whether the presence of silicic acid is sufficient to counteract the adverse effect of aluminium on mineralization is as yet unclear.

TABLE 4 Effect of ions on calcium phosphate precipitation

Addition (1mM)	Phases present (from X-ray diffraction)
Control	$CaHPO_4.2H_2O + Ca_8H_2(PO_4)_6$ (t)
$Si(OH)_4$	$CaHPO_4.2H_2O + Ca_8H_2(PO_4)_6$ (t)
$Na_2H_2P_2O_7$	Non-crystalline material
Al Cl_3	Non-crystalline material + $CaHPO_4.2H_2O$ (t)

t, trace.
[Ca], 2.5 mM; [PO_4], 6.6 mM in 80 mM KCl at 17 °C, initial pH 7.4, aged 24 hours, filtered and dried at 80 °C.

Conclusions

In higher animals, the only established effect of silicon deficiency is on hard and soft connective tissue, and in particular on osteogenesis. Whether the observed effects are related to changes in bone organic matrix or the mineral phase is unclear. The silicon present in tissue must derive from silicic acid and in this study we have drawn attention to the interactions of silicic acid with metal ions, an area apparently little considered in the biological context.

Silicic acid, being a weak acid (pK_1 9.8), interacts only with basic metal ions; therefore, at physiological pH, calcium and magnesium are excluded. We briefly investigated silicic acid–Mn^{3+} chemistry, since there are several Mn-dependent enzymes in mucopolysaccharide synthesis, but found no reactions of interest.

There are interactions with Fe^{3+} and silicic acid around neutral pH that are of interest to soil scientists and water chemists. We have attempted to assess the relevance of these to living systems. It seems unlikely that silicic acid plays any role in the detoxification of haemosiderin. It is also unable to suppress the formation of polymeric Fe–O species and is therefore not a candidate ligand in the labile iron pool. However, the balance between complex formation and precipitation is such that aggregation is limited by stable colloidal particles and this effect has explained some early observations of silicic acid aiding iron availability to plants.

The observation that silicic acid occurs in key locations in growing bone and the now established deleterious effect of aluminium in bone have prompted us to study the interactions of the two, especially in view of their unique affinity in aqueous, colloid and solid-state chemistry. Preliminary experiments have indeed suggested that silicic acid can counteract deleterious effects òf aluminium (and possibly other metals) and this raises the possibility that many of the reported effects of silicon can be explained by such a mechanism̃, with silicon having no independent role.

The feature of silicon deficiency constantly emphasized is collagen synthesis

and structure. This directed our attention to the vital co-factors and, in particular, to the mobilization and availability of Fe. Our results suggest that Al species can compete for Fe binding sites (e.g. in prolyl hydroxylase), causing reduced activity. The role of silicon, we suggest, is to interact (as silicic acid) with Al species (e.g. $Al(OH)^{2+}$), with the resulting aluminosilicate species having altered binding characteristics and, specifically, being unable to mimic Fe. In the absence of silicon (or in an excess of Al) collagen synthesis and structure are adversely altered and this could account for the observed effects of Si deficiency: bone organic matrix changes, reduced weight gain, reduced rates of wound healing, etc.

Clearly much more work is required, since the topic is currently of interest in determining the true relationship of aluminium and silicon to Alzheimer's disease (Krishnan & Crapper-McLachlan 1985) and in understanding the toxicity of Al^{3+} to fish, in which the speciation of aluminium and aluminosilicate ions are important topics.

Acknowledgements

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DISCUSSION

Werner: It is interesting to compare your data on human tissue with effects in plants. In a certain concentration range silica reduces manganese toxicity by two orders of magnitude, which is significant. This effect is apparently different from the effects of silica as a functional nutrient in some plants, whereas much higher concentrations of silica are needed to interfere with manganese toxicity. There is an interesting relationship, but it depends very much on the concentration.

Birchall: It is probable that aluminium is always present. Probably a balance between the two is necessary. In other words if you eliminate silicon you observe the toxicity of aluminium. An effect of silicon (silicic acid) in reducing the toxicity of Mn was described by Okuda & Takahashi (1962).

Carlisle: I have data that indicate that aluminium is an essential element (Carlisle, unpublished work 1985). In certain tissues I believe that both aluminium and silicon are essential.

Birchall: That may be so, but my point is that we can account for most of the observations on bone and collagen formation. Aluminium appears to inactivate prolyl-4-hydroxylase. It is interesting that when an aluminosilicate ion is

formed, the aluminium no longer looks like a metal; its binding characteristics are altered and it no longer deactivates the enzyme.

Carlisle: I believe that silicon and aluminium work synergistically in certain tissues and that both are essential elements in these tissues. I also believe that aluminium and silicon are under homeostatic control.

Birchall: The next obvious thing to try is tyrosine hydroxylase.

Edwardson: The total content of aluminium in the brain in Alzheimer's disease may not be very different to that in age-matched controls. However, the intracellular and extracellular distribution is entirely different, so total content means virtually nothing. At least some of the neurons which seem to be selectively vulnerable in Alzheimer's disease contain large amounts of the vitamin-D induced calcium-binding protein (K. Bainbridge, personal communication). Aluminium binds *in vitro* to calcium-binding proteins. If there was some initial complexing of aluminium with particular proteins, presumably this would still allow subsequent reaction with silicic acid?

Birchall: I believe the important point to be that the binding characteristics of aluminium species—possibly $[Al(OH)]^{2+}$ and $[Al(OH)_2]^+$, see Brown & Sylva (1985)—will be greatly altered by the formation of aluminosilicate ions and there is a peculiar affinity for aluminium species and silicic acid in aqueous solution (Iler 1979, p 193 and p 80). We show that aluminium inhibits prolyl hydroxylase and this suggests that it is competing for the iron binding site at which iron is bound loosely. Silicic acid—which has no effect on the enzyme itself—seems to prevent this, either because an aluminosilicate ion is formed with altogether different mass/charge, or because a colloid is precipitated. Willey (1975) has shown that only 1 p.p.m. SiO_2 is required to precipitate 1 p.p.m. Al_2O_3 from solution. Equally, I would expect that the interaction of aluminium species and silicic acid would reduce the ability of aluminium to compete for calcium binding sites. Silica and alumina have remarkably opposite effects. Iler (1979, p 763) has noted that the adsorption of proteins on quartz was prevented when as little as 5% of the surface was covered with aluminosilicate ions. In answer to your specific question, if aluminium became bound to a protein, I can envisage *subsequent* interactions with silicic acid. I suggest that the *prior* combination of aluminium species and silicic acid would alter binding.

Werner: You and Dr Carlisle both showed the local concentration of silicon going up and down in bone. If you used organisms without silicon in the diet, if our conclusion is valid, aluminium should replace it at this spot. Has aluminium been measured at the same spot?

Birchall: Did you scan simultaneously for aluminium, Dr Carlisle?

Carlisle: Yes; aluminium was present in very minute amounts compared to the silicon at the same spot.

Williams: It is not too easy to measure the aluminium with your method, is it?

Carlisle: No, but the aluminium was definitely at the same spot, as shown by X-ray micrographs.

Henrotte: Loeper et al (1984) recently published some experiments showing that silicon reduced lipid peroxidation in the rabbit aorta. How does that fit into your scheme, Dr Birchall?

Birchall: That was one reason why we did our study. That is one hypothesis—that silicon is somehow protecting against peroxidation, in particular by haemosiderin micro-deposits. Our conclusion is that it is highly unlikely that it is.

Carlisle: That is interesting. We find the aorta to be relatively high in silicon content compared to the other tissues.

Hench: You compared the isoelectric points of silica with those of alumina. We have been studying alkoxide-derived amorphous alumina gels and the ageing effects on these gels. We see variations in isoelectric points as a function of the ageing time of the amorphous gels that can vary by three or four pH units. Could the protection effect that you are proposing have a time-dependent aspect which is related to variations in the effective isoelectric point of a small complex like these gels?

Birchall: Solid aluminosilicates will have a zeta potential dependent on the Al:Si ratio—all the way from positive when there is little silicic acid to negative when there is excess. The ability of organic molecules to adsorb on such solids will depend on the surface charge. The binding characteristics of small (dissolved) aluminosilicate ions will also depend on the Al:Si ratio.

Hench: The point of zero charge changed three or four pH units as a function of time with the alterations in the structures of the amorphous alumina gels.

Espie: The classic colloid science experiment would be to change the zeta potential of alumina by adding silicic acid. You get a complete charge reversal. Your ageing effect on fresh alumina gel going to anhydrous alumina would produce reorganization and contamination of that surface which could alter the surface charge.

Dobbie: In clinical research some investigators are beginning to question whether silicon is a protective element. Perhaps we are becoming too caught up with the test tube. I would like to think in more holistic terms about what is happening to the relative concentrations of aluminium and silicon in interstitial fluid and plasma. This is of course controlled by absorption and excretion. The natural relative balance between silicon and aluminium is a phenomenon with which many enzyme systems have evolved from the earliest forms of life. This balance has been grossly altered in certain situations, particularly in medicine. In patients with damaged kidneys given large doses of aluminium hydroxide gross differences between aluminium and silicon can be shown by atomic absorption spectroscopy which are totally unknown in nature. Animals which excrete certain trace elements in their urine are not usually kept alive after renal failure, and aluminium was of no interest to the medical profession until dialysis began. My prediction is that the levels no longer run at the normal side-by-side level in these abnormal circumstances.

Carlisle: It is analogous to what happens with calcium and zinc. If an animal is

on a diet that is borderline with respect to zinc and additional calcium is added to the diet, the animal will become zinc-deficient.

Williams: Aluminium and silicon seem to go together in inorganic chemistry but in organic chemistry they are as different as chalk and cheese. The aluminium will always be picked up by things like these dihydroxyphenols with a very high binding constant. Aluminium always gets removed by reagents like phosphate but silicon won't get stripped out by phosphate. In an organic system we cannot expect aluminium and silicon to behave in the same way. They are very different from each other.

Carlisle: There must be an efficient homeostatic control. In some animal tissues silicon and aluminium appear to have little relationship to one another. Then in certain other tissues we see this constant relationship which is even present on a very low silicon–aluminium diet.

Williams: Chemistry in an organic environment is not like inorganic hydrolysis. Aluminium is very good at binding to oxalates and so on, and it can bind them at quite high pH. Silicon has quite a different chemistry. Aluminium is really a metal, while silicon is on the borderline with non-metals.

Farmer: The possible species of soluble aluminium in blood and serum are of interest here. Monomeric inorganic species will exist in some sort of equilibrium with, possibly, polymeric hydroxyaluminium species, organic complexes, and silicate complexes. The formation of an aluminosilicate complex requires the presence of minimum amounts of both aluminium and silicon as simple monomeric hydroxycomplexes. In pure systems with only gibbsite present, its solubility is a minimum (2×10^{-8} M-Al) at pH 6.0, and increases to about 5×10^{-8} M at blood pH (7.0). At this pH, the dominant monomeric species is the $Al(OH)_4^-$ anion (May et al 1979). If soluble aluminium in blood exceeds this level, it must be present as organic complexes, or polymeric hydroxycomplexes, or a polymeric aluminosilicate. Of these, organic complexes seem the most probable, as hydroxyacids of the citric acid cycle, for example, form strong complexes with aluminium, much stronger than silicic acid. For proto-imogolite to form, silicon in solution must exceed 1 mg/l even to prevent the preferential formation of simple hydroxyaluminium polymers. Iler (1979), in reviewing published data for silicon concentrations in blood and serum, favours recent findings of 0.2–0.5 mg Si/l, rather than earlier reports of around 4 mg Si/l. Aluminium silicate complexes or precipitates should not form at the lower silicon concentrations, but could at the higher.

Hench: In the first stages of bone repair the pH is low, around 5.4. Thus soluble silicon as a stable species will be in very low concentrations. In normal bone repair, the pH gradually increases all the way up to pH 9.4, which can result in a larger concentration of soluble silicon and hydrated, mineralized calcium phosphate salts (hydroxyapatite). Dr Birchall's results suggest that chronic disunion may involve an imbalance in the silicon/aluminium concentra-

tions which prevent this normal sequence of mineral precipitation and crystallization.

Williams: Another problem in this chemistry is that in any biological structure there are phosphates everywhere, at 3 mM. These could clean the aluminium out of the whole system. There can't be much aluminium floating around free.

Espie: Given free aluminium, the phosphate would generate insoluble aluminium phosphates immediately. However, there is never any free aluminium in plasma since it is preferentially bound up with transferrin (Trapp 1983) with an estimated stability constant of 10^{24}.

Birchall: Aluminium could not possibly compete with iron in haem-dependent enzymes but, in view of our result with aluminium and prolyl hydroxylase—in which iron is bound *loosely*—we should pay attention to other enzymes in which the binding constant for iron is low (the hydroxylases for example, some of which are basic in neurochemistry). It is suggestive that Al is bound in transferrin. Does aluminium interfere with enzymes and does silicic acid prevent inhibition? There is a flux of aluminium in the body with 15 μg being excreted per day and adsorption increased in Ca and Mg deficiency and possibly influenced by parathyroid hormone. We cannot easily dismiss competition between iron and aluminium and I would want to bring calcium into the argument. We know aluminium to be deposited in brain and in bone with dramatic effects—so it is present and mobile.

Williams: The hydroxyproline enzyme is only one of the cases where the free iron pool is in equilibrium with the enzyme. About 20 enzymes are just as sensitive to free iron.

Birchall: Nobody has looked at that yet.

Williams: Looking after iron is a major job for biology. Biology won't be fooled by aluminium.

Edwardson: Transferrin is fooled; 50% of the plasma aluminium in normal individuals is bound to transferrin.

Williams: That is because transferrin is not overloaded with iron. On the cell surface there is a receptor for transferrin and it will accept aluminium in transferrin, at this stage. The membrane with the receptor breaks and forms a small vesicular system in the cell. This vesicular system is a sort of mini-lysosome and it holds the iron beautifully. The iron probably comes out of the transferrin complex and is mobilized in the cell in the form of iron citrates and so on, entering the pool with ferritin. If aluminium follows it in, then it remains as trivalent aluminium. However, when the cell handles iron it switches its valence state from FeIII to FeII. It can manipulate iron beautifully in this way but it can't touch aluminium. It is always stuck on the proteins or elsewhere as Al(III). If the transferrin protein keeps its aluminium in the above vesicle it will just reject the Al(III) when the protein goes back to the membrane.

Birchall: I don't believe that we know enough to be dogmatic.

Espie: You are invoking a mechanism there in which transferrin iron is reduced. There is an equally acceptable mechanism that says that acidification in the lysosome is sufficient to release iron from transferrin. Aluminium would also be released.

Williams: If you get acidification the redox potential drops, which helps a reductive pathway.

Espie: That is still a debatable point.

Williams: Inside the cell the redox potential drops to way below 0.0 volts. There is no obvious reason to suppose that aluminium can interfere directly with any iron enzyme site. That has to be confirmed.

Birchall: In other words you now have to find another explanation for our results.

Hench: One of the most important problems facing the human species today is the stable immobilization of 400 million gallons of high level radioactive waste. The world decision on that is to use a silicon-based matrix in the form of a soda borosilicate glass for storage purposes. But the behaviour of those glasses in contact with groundwater is subject to all the pH variations that Dr Farmer has talked about. The data for long-term predictions are mainly based on glass leaching in distilled water. The total storage system often includes iron or steel canisters which hold the glass. It has been discovered that the leaching that takes place depends on variations in iron silicate complexes. When nuclear waste glasses are leached alone, in distilled water or in geological conditions, saturation of silicate species occurs and the glass remains in a stable condition. But in the presence of iron, silicate complexes appear as colloids and those colloids are potentially transportable to the biosphere, as reviewed by Birchall. When the glass leaches in this kinetic mode it releases radioactive elements which can complex with those same iron silicate species. Then the system is less able to protect the biosphere from the waste. That has to be engineered out by eliminating labile iron from the waste package. Even more important, we need to understand how those complexes form and how they can be irreversibly sorbed within the waste storage barriers.

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Aluminosilicates and the ageing brain: implications for the pathogenesis of Alzheimer's disease

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Abstract. Senile plaques are a neuropathological feature of the ageing brain and consist of abnormal neuritic and glial processes surrounding an extracellular core of material with fibrillary ultrastructure. Present at low densities in the cerebral cortex of most aged individuals, they occur in large numbers in Alzheimer's disease, the major form of senile dementia. Energy-dispersive X-ray microprobe analysis of isolated cores and plaques *in situ* from patients with Alzheimer's disease or Down's syndrome and from normal controls has shown co-localization of high concentrations of aluminium (4–19%) and silicon (6–24%) at the centre of the core. The presence of these elements as aluminosilicates has been confirmed using solid-state ^{27}Al nuclear magnetic resonance. These findings provide a link with the other major neuropathological feature of Alzheimer's disease, the neurofibrillary tangle-bearing neurons, where high intracellular levels of Al and Si have also been reported. The focal deposition of these elements may be an early and essential factor in the pathogenesis of Alzheimer-type changes, reflecting an increased exposure to aluminium.

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Ageing of the brain in normal individuals beyond 65–70 years is commonly associated with a mild degree of cerebral atrophy and changes in microscopic appearance. Silver staining reveals an increase in senile plaques and, in the hippocampus, neurofibrillary tangles, two neuropathological features which are of clinical interest since their widespread distribution at high densities is a diagnostic feature of Alzheimer's disease (Tomlinson 1984). This disorder is characterized by severe, progressive impairment of intellectual function and accounts for over half of all cases of senile dementia. Histologically, senile plaques consist of an extracellular core of amyloid surrounded by abnormal neuronal and glial processes, the whole structure ranging from 15–200 μm in diameter (Fig. 1A). The intensely argyrophilic plaque core has a fibrillary
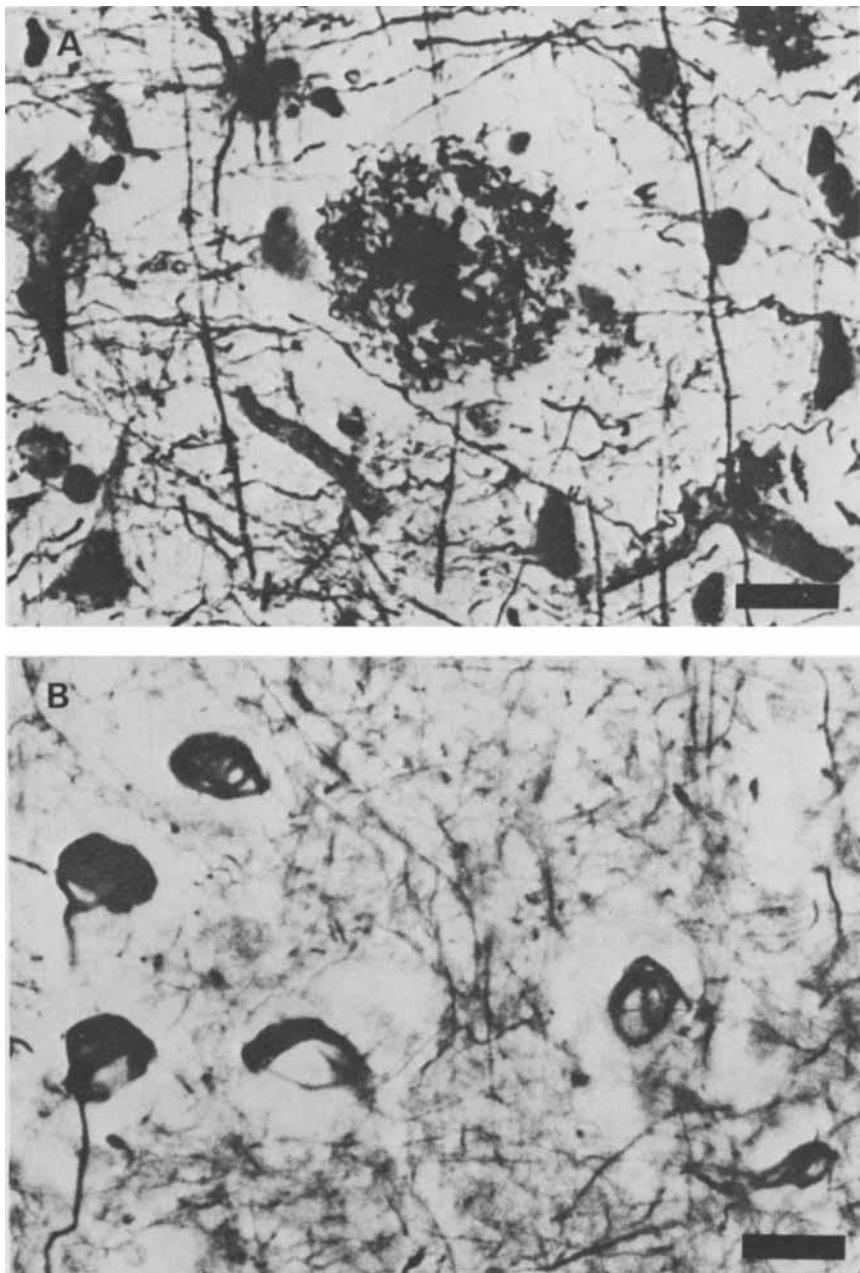
FIG. 1. Shows the appearance of (A) a senile plaque, (B) neurofibrillary tangle-bearing neurons in a silver-stained section of the cerebral cortex from a patient with senile dementia of Alzheimer type. Calibration bars represent 15 μm.

ultrastructure and is usually considered to consist of protein in β-pleated sheet configuration. Neurofibrillary tangles consist of dense intracellular deposits of protein which also stain heavily with silver (Fig. 1B) and are largely in the form of paired, helically twisted filaments.

Recently, we have shown that several endogenous brain peptides are capable of forming fibrillary polymeric aggregates with an ultrastructure and staining properties resembling plaque core amyloid (Oakley et al 1981). The disordered neuronal processes involved in plaque formation are, in many cases, derived from axons or presynaptic terminals since they contain neurotransmitter storage vesicles. Thus, peptide transmitters such as substance P or somatostatin could possibly contribute to the formation of extracellular fibrils found in plaque core amyloid. We have attempted to isolate and characterize the chemical constituents of senile plaque cores isolated from the cerebral cortex of patients with senile dementia of the Alzheimer type.

Presence of aluminosilicates in the senile plaque core

A modification of the technique used by Allsop et al (1983) has been used to isolate the core material. This technique relies on the extreme resistance to disaggregation of the amyloid core in senile plaques. Samples of grey matter from frontal or temporal cortex were homogenized in 0.1 M-phosphate buffer, sonicated and then incubated with protease (subtilisin BPN) for 3 h at 37 °C to digest the tissue. The material was then treated with 2% sodium dodecyl sulphate overnight at 4 °C to disaggregate proteins. Subsequent treatment included incubation in 4 M-hydroxylamine hydrochloride at 37 °C to remove collagen, and isolation of the residual cores by sucrose density gradient centrifugation (Candy et al 1986). This technique yielded roughly spherical, particulate material, corresponding in size to senile plaque cores (Fig. 2A) and having the ultrastructural and the staining properties of plaque cores *in situ*. When the isolated cores were spattered by centrifugation (160 000 g 30 min) on to an electron microscope grid and stained with 1% uranylacetate, fibrillary material with a subunit diameter of about 3 nm was seen (Fig. 2B). The isolated cores were resistant to disaggregation by a range of solvents including 6 M-guanidine, 6 M-urea, 100% acetonitrile, 98% trifluoracetic acid and 5% sodium dodecyl sulphate. They had the classical staining properties of plaque cores *in situ*, including silver impregnation, apple-green birefringence with Congo Red under polarized light, and crystal violet metachromasia. Surprisingly, the protein content, as judged using a number of procedures (Lowry assay, fluorescamine assay, hydrolysis with 6 M-HCl and amino-acid analysis, infrared analysis) was always less than 5% and sometimes as low as 1%.

In attempts to determine the composition of this isolated material, plaque cores were embedded in epon and sectioned (3–4 μm). Elemental analysis was done using a Jeol JSM 35 scanning electron microscope and Link 860
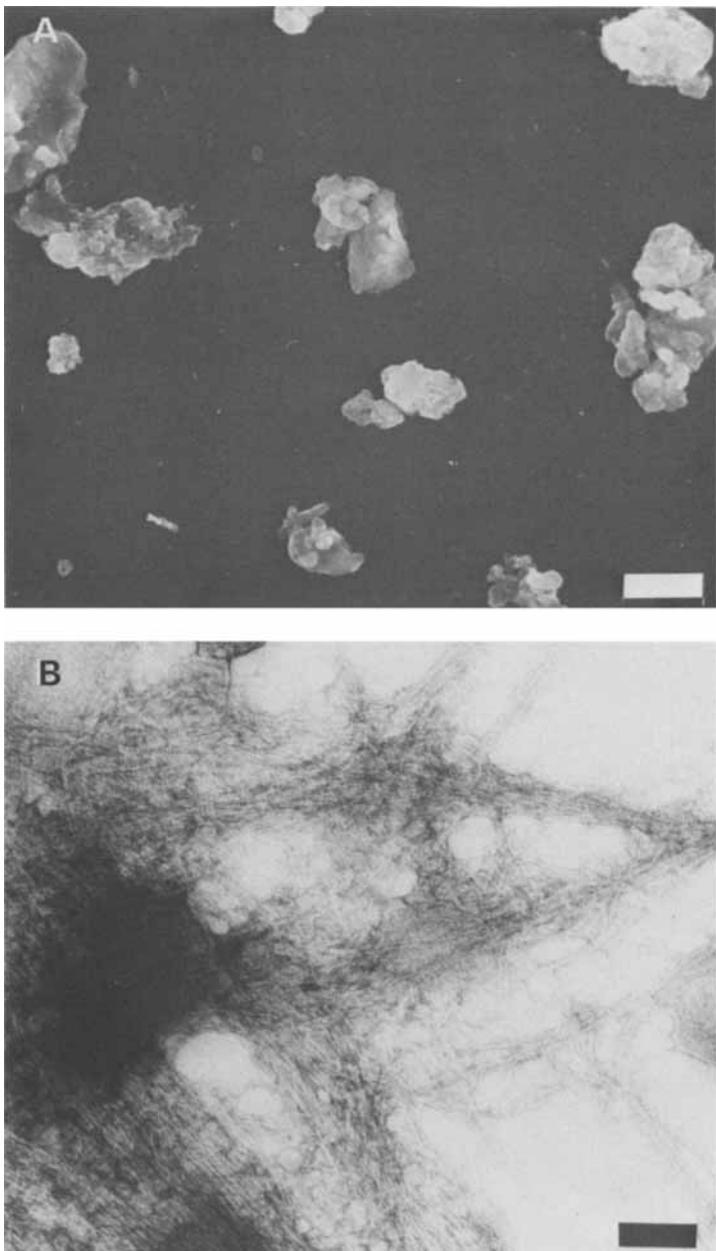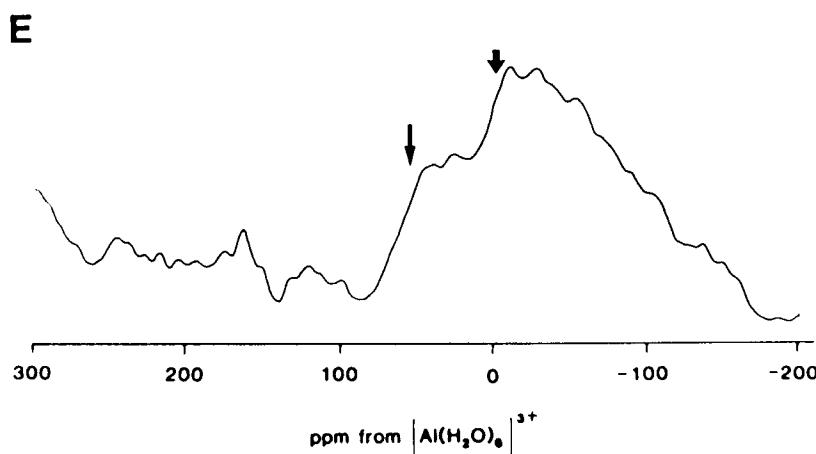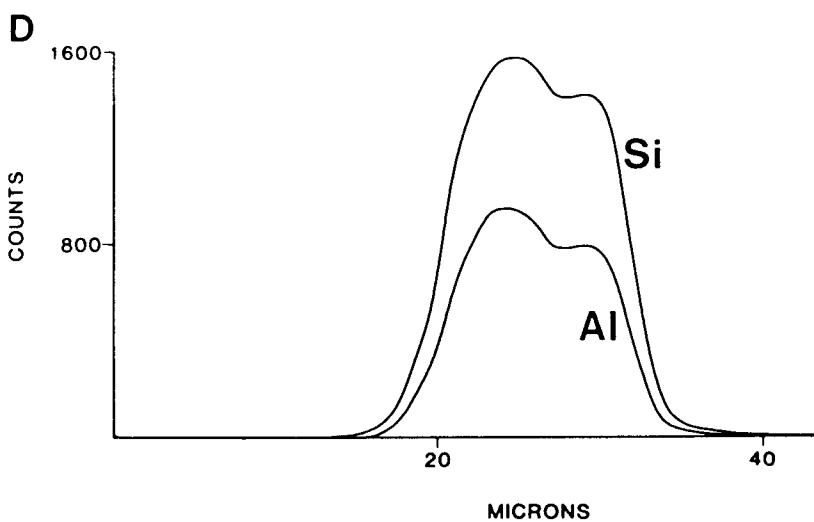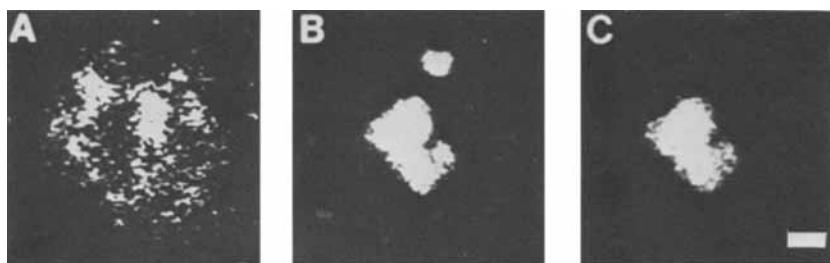
FIG. 2. (A) Scanning electron microscope of senile plaque cores isolated from the cerebral cortex of a patient with senile dementia of Alzheimer type. Calibration bar represents 10 μm. (B) Transmission electron micrograph of an isolated senile plaque core after centrifugation on to an electron microscope grid coated with formvar and carbon and negative staining (1% uranyl acetate). The fibrillary nature of the core at its periphery can be seen. Calibration bar represents 83 nm.

energy-dispersive X-ray microanalytical system (EDS) with digimap facilities. This procedure revealed that co-localization of aluminium and silicon was a consistent feature of all isolated plaque cores (Candy et al 1984, 1985, 1986). Whereas some constituents such as sulphur (Fig. 3A) are distributed throughout the plaque core, including the periphery, Al and Si (Fig. 3B, C) are more discretely localized in the central region. Quantitative analysis of these elements in epon-embedded sections through the central core region gave values ranging from 4 to 19% for Al and 6 to 24% for Si, in contrast to other elements which were much less abundant (percentage values were: P, 0.15; S, 1.5; Mg, 1.14; Fe, 3.11; Zn, 1.26; Ca, 0.15; K, 0.21; values obtained for a plaque core; Al, 18.9 and Si, 21.9). Line scans of the elemental distribution within the core showed a coincident distribution of Al and Si (Fig. 3D) and suggested the possibility that these elements are present as an aluminosilicate. This has been confirmed by solid-state nuclear magnetic resonance (NMR) with magic-angle-spinning (MAS), a technique which can determine the coordination state of Al in very small amounts of material (Fyfe et al 1982). The ^{27}Al MAS-NMR spectrum obtained from approximately 1 mg of isolated plaque cores shows the presence of octahedrally and tetrahedrally coordinated aluminium (Fig. 3E) which is typical of amorphous aluminosilicate (J. Klinowski, T.A. Carpenter & J.M. Thomas, unpublished).

The coincident distribution of Al and Si within plaque cores was also shown for plaques *in situ* using cryostat sections of formalin-fixed, silver-stained cerebral cortex from five senile and two presenile cases of Alzheimer's disease which had been clinically and neuropathologically assessed. Over 75% of all plaques studied by EDS analysis showed coincident distribution of Al and Si; this procedure would not be expected to yield a positive result in every case since penetration of the electron beam is limited and masking by silver deposits is likely to occur. Similar analyses were done using unfixed, unstained frozen sections from two senile cases of Alzheimer's disease. Foci corresponding in size to plaque cores and exhibiting coincident distribution of Al and Si were observed (Fig. 4). Similar results on the co-distribution of Al and Si have been found for senile plaques in the brains of intellectually normal elderly individuals (Fig. 5A), where they frequently occur at a low density, and also in Down's syndrome (Fig. 5B) where classical Alzheimer-type changes involving

FIG. 3. (A), (B) and (C) show the distribution of S, Si and Al in a section (3–4 μm) through an epon-embedded isolated senile plaque core. It can be seen that silicon and aluminium have a similar discrete localization in the central region of the plaque core. The calibration bar represents 4 μm. (D) shows the coincident distribution of Si and Al in a line scan (X-ray signal intensity plotted against distance) across the isolated plaque core shown in (A), (B) and (C). (E) ^{27}Al MAS NMR spectrum obtained from isolated plaque core material. Two coordination states of Al are present: 6-coordinated (octahedral; right-hand arrow) and 4-coordinated (tetrahedral; left-hand arrow).
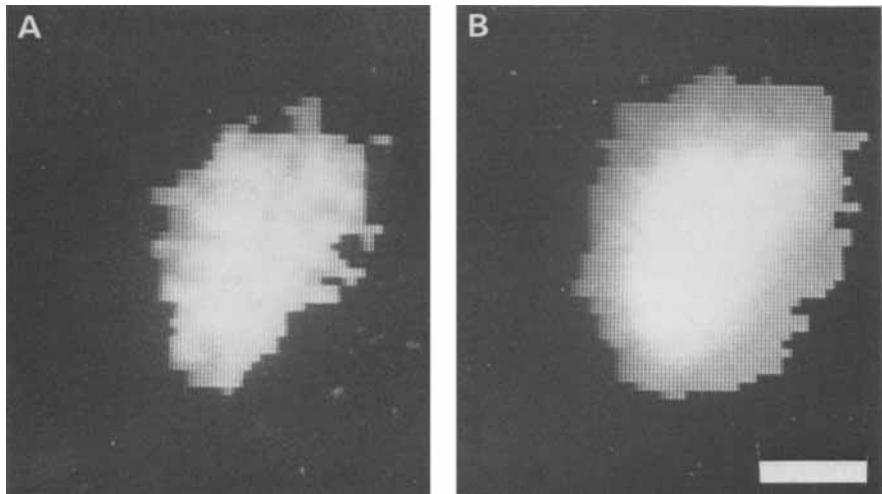
FIG. 4. The presence and co-localization of (A) Al and (B) Si in a presumed senile plaque core in an unfixed, unstained cryostat section from the cerebral cortex of a patient with senile dementia of Alzheimer type. Calibration bar represents 5 μm.

both plaques and tangles are invariably found *post mortem* in individuals over 30 years of age (Burger & Vogel 1973).

These findings indicate that aluminosilicates are a consistent component of senile plaque cores and raise novel questions regarding the pathogenesis of these structures. The low protein content and the high content of Al and Si in the central region of isolated plaque cores do not immediately seem consistent with their fibrillary ultrastructure and staining characteristics, which resemble those of amyloid protein deposits. We have pointed out elsewhere (Candy et al 1985) that some naturally occurring aluminosilicates such as imogolite exist in fibrillary form and have both staining and ultrastructural features similar to those shown by isolated core material. Application of MAS-NMR in relation to ^{29}Si and ^{13}C should help to resolve the identity of the aluminosilicate and determine the nature of the other non-protein organic constituents.

Aluminosilicates and plaque formation

A sequence of events in senile plaque formation was proposed by Terry & Wisniewski (1970), based on the study of plaques presumed to be at different stages of development. According to these workers, the earliest phase consists of several swollen neuritic processes without an amyloid core. At a later stage a core surrounded by many neuritic processes is present, while at the end phase only the core remains. In this scheme, amyloid deposition is a secondary
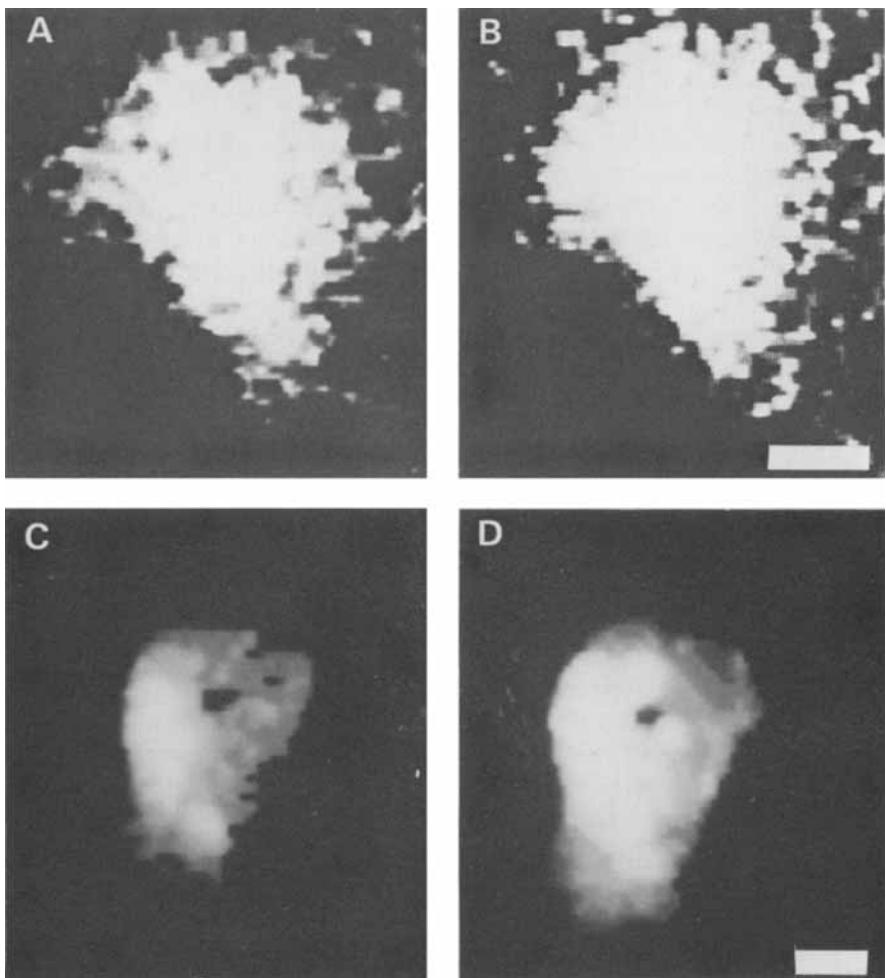
FIG. 5. The presence and co-localization of (A) Al and (B) Si in a senile plaque core *in situ* in the cerebral cortex of an elderly, mentally normal patient. Calibration bar represents 2 μm. *In situ* co-localization of (C) Al and (D) Si in a senile plaque core in the cerebral cortex of an elderly patient with Down's syndrome. Calibration bar represents 3 μm. Maps of the elemental distribution in the senile plaques were produced in silver-stained, formalin-fixed, cryostat sections.

process. However Rudelli et al (1984) have recently reported that, in subcortical white matter, amyloid deposits can develop in the absence of neuritic elements. Furthermore, in non-demented patients, amyloid-like material has been reported to occur in the absence of neuritic processes in the cerebral

cortex (Ulrich 1985). These findings suggest that amyloid may be the initiating factor in the formation of senile plaques. Early plaques, while lacking an obvious core, may still reflect the focal accumulation of amyloid precursors or aluminosilicates.

In view of the evidence that core material may be an initiating factor in plaque development, it is important to consider the possible role of aluminosilicate in this respect. Marinesco (1928) first proposed that the deposition of an argyrophilic substance elicited plaque formation, and the cation binding properties of aluminosilicates are consistent with this hypothesis. Immunostaining procedures have shown that albumin and immunoglobulins may be present in the plaque core but their presence is considered to be non-specific, reflecting the 'sticky' nature of the amyloid core. However, several factors point to the possibility that the deposition of aluminosilicates may be an essential stage in plaque formation. Their localization at the centre of the core suggests an early involvement in plaque formation since secondary deposits would more likely be peripheral or homogeneous throughout the core. Also, if the presence of Al and Si simply reflects non-specific binding by pre-existing core material it seems likely that other elements such as Ca, Mg, Fe, S and P would be present to the same extent, but this is not the case. The presence of Al and Si in plaques from normal elderly brain, and from patients with senile or presenile Alzheimer's disease or Down's syndrome, indicates that aluminosilicate is a constant component of plaques wherever they occur. Even if the deposition of aluminosilicate is secondary, the space-occupying effect of these bulky inorganic deposits and the chemically reactive nature of aluminosilicates make it highly probable that they would contribute to the disruption of adjacent neuronal and glial processes.

The mechanism of aluminosilicate formation and deposition in the plaque core is at present unknown. Preliminary observations on the distribution of Al and Si in plaques from patients with Down's syndrome indicate that there may be some dissociation which is not typically seen in Alzheimer's disease, i.e. there are patchy concentrations within the core of one element without the other. It is not known whether the local entry, binding and accumulation of Al precedes that of Si or whether both occur in parallel. While Si is much more abundant than Al in blood and many body tissues, levels of Si in the cerebrospinal fluid are lower than those in plasma (Austin et al 1973, Hershey et al 1984). There has been previous speculation that Al in the nervous system may be complexed with Si but it has been assumed that aluminosilicate would represent a biologically unreactive state of the metal. However, aluminosilicates have several properties including proteolytic catalyst activity which could well play a role in secondary pathogenesis. Recent attention has focused on amyloid protein as an initiating factor in plaque formation. It is at least possible that the protein reflects a secretory response to the presence of aluminosilicate, in the form of a mechanism for isolating the latter.

Aluminium and the pathogenesis of Alzheimer's disease

The significance of aluminium in the pathogenesis of Alzheimer's disease has been a controversial issue for well over a decade. Thus, the finding that aluminosilicates are a consistent feature of the senile plaque core is of particular interest, since Duckett & Galle (1976) reported the presence of aluminium in senile plaques and Nikaido et al (1972) observed increased levels of silicon in isolated senile plaque cores. In addition Perl & Brody (1980), using scanning electron microscopy with X-ray spectrometry, have shown that raised intracellular levels of Al occur in neurons with neurofibrillary tangles—the other major neuropathological feature of Alzheimer's disease. These workers also showed that Si was present in neurons with neurofibrillary tangles. Interest in the role of Al in Alzheimer's disease stemmed from observations that injections of Al salts in the rabbit could induce the formation of neurofibrillary tangles, although the experimental tangles do not contain the paired helical filaments typical of the human disease. Aluminium treatment of cats and rabbits has been shown to produce changes including disruption of short-term memory, disorganization of EEG slow-wave activity, focal neurological deficits and seizures (Crapper 1976). Industrial exposure to high levels of Al has been reported to produce CNS damage in rare cases but the most significant example of the neurotoxic effects of this metal in humans is the dialysis encephalopathy syndrome (Wills & Savory 1983). Patients in renal failure are exposed to increased amounts of aluminium through treatment with phosphate-binding gels which contain Al and, in some areas, because of high levels in the water used for dialysis. Because of renal failure this Al load is not excreted, and the neurological consequences of this chronic Al intoxication include speech defects, dementia and seizures. Moreover brain levels of Al in dialysis dementia are greatest in patients showing neurological involvement.

It has also been claimed that brain tissue concentrations of Al are raised in Alzheimer's disease, in some cases to levels which are cytotoxic in animals *in vivo* and cultured human neurons *in vitro* (Crapper-McLachlan & De Boni 1980). However, other workers have failed to find significant increases in Al in the brain of demented individuals although they have shown a general age-related increase (McDermott et al 1979). Differences in case selection and brain sample size, together with the large variation in Al levels even in elderly individuals with apparently normal intellectual function, may contribute to this discrepancy. Although this issue has not been resolved, there is a general view that the involvement of Al in Alzheimer's disease is non-specific or may reflect an increased uptake by degenerating neurons. However, the work of Perl & Brody (1980) and our own studies (Candy et al 1984, 1985, 1986) show that, irrespective of total brain load, plaque and tangle-related Al is massively increased in Alzheimer's disease since these lesions are much rarer in normal subjects. In severe cases of Alzheimer's disease vast numbers of plaques and

tangles may be present and the distribution and form of Al in such cases probably differ greatly from those in non-demented cases, even if the total content does not differ.

While the accumulation of aluminosilicates could induce the structural changes in plaque formation, the factors which initiate such local deposition are not known. There is some evidence for an impaired blood–brain barrier in Alzheimer's disease, and genetic, viral, autoimmune, toxic or other factors could contribute to such failure. Recent work has drawn attention to the possible contribution of abnormal calcium homeostasis in this respect.

Aluminosilicate formation and brain calcium homeostasis

Neurofibrillary tangles occur in several conditions, including a disorder found with a high incidence in foci on Guam and in other Pacific areas, where symptoms of Parkinson's disease coexist with dementia and motor neuron disease. Tangle-bearing neurons in this disorder contain high levels of aluminium and calcium (Garruto et al 1984). Failure to find any evidence for a genetic or infectious causation has drawn attention to environmental factors. The populations at risk suffer from a chronic deficiency of calcium and magnesium and are exposed to high levels of aluminium and manganese. Although cortical and subcortical senile plaques are lacking in the Parkinsonian dementia complex of Guam, studies of this disorder raise the question of whether the uptake of Al and formation of aluminosilicates in Alzheimer's disease may also be related to some chronic abnormality of mineral homeostasis. Experimentally induced Ca–Mg deficiency in rats has been reported to increase levels of Al in the CNS (Yase 1980), as has injection of parathyroid hormone (Mayor et al 1980). A significant proportion of the elderly population is in negative calcium balance, with decreased Ca^{2+} absorption, reduced vitamin D hydroxylation, and increased levels of parathyroid hormone. Studies to date have failed to show changes in blood levels of parathyroid hormone, Ca or Al in Alzheimer's disease. However, it is difficult to draw conclusions from these negative findings; in senile or post-menopausal osteoporosis, where progressive loss of Ca is evidenced by a diminishing bone mass, blood levels of Ca or the calcium-regulating hormones, parathyroid hormone and calcitonin, do not usually change.

Little is known of the local regulation of calcium homeostasis in the brain but changes in Ca^{2+} transport systems have been linked to age-related and pathological processes involving neurotransmitter systems, energy metabolism, axonal flow, tangle formation and cell death (Khachaturian 1984). Aluminium is known to bind with many of the macromolecular substrates of these Ca^{2+}-mediated processes (Boegman & Bates 1984) and it may be relevant that, for example, the magnocellular neurons of the nucleus basalis of Meynert which appear particularly vulnerable in Alzheimer's disease stain intensely with

antibodies raised against the calcium-binding protein, CBP (K. Baimbridge, personal communication). A broad hypothesis suggests that the cause of Alzheimer's disease is multifactorial, involving a primary lesion, genetic or acquired, which predisposes affected individuals to breakdown of the blood–brain barrier for Al with ageing. The rate of Al deposition and aluminosilicate formation would, in addition, be determined by the Ca^{2+} status of the individual and the extent of environmental exposure to aluminium. This hypothesis predicts that there should be marked geographical variation in the incidence of Alzheimer's disease and related disorders, and recent epidemiological evidence supports this view (Gardner et al 1984). Thus, for example, there is a significant difference between the neighbouring counties of Northumberland and Durham in the standardized mortality rates for dementia, as measured from death records for the period 1968–78. Overall rates in Northumberland are higher for both men and women (231% and 208%) in comparison with Durham (79% and 98% respectively). The patterns of distribution for dementia and also for motor neuron disease and Parkinson's disease show geographical clustering which cannot be explained readily by local differences in the practice of death certificate reporting or the location of hospitals with long-stay psychogeriatric wards (D.J. Barker and J.A. Edwardson, unpublished data). Possible factors which could account for such regional differences are currently being investigated, including the natural mineral content of soil and water and the use of aluminium in the treatment of drinking water.

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DISCUSSION

Volcani: Austin (1978) described the distribution of silicon in the brains of patients with Alzheimer's disease. Have you looked at that?

Edwardson: Data from studies on the disorder in Guam (Perl et al 1982) don't show a significant increase of silicon in tangle-bearing neurons, which contain high concentrations of aluminium. Our study was exclusively on the extracellular deposits found in patients with senile plaques where there is parallel distribution of aluminium and the silicon.

Volcani: Austin pointed out that there is, uniquely, selective accumulation of silicon in the amyloid bodies in patients with dementia. The brains of non-diseased people have the same total concentration but the distribution is different.

Edwardson: Reports by Crapper et al (1973) and other groups suggested that there was a specific increase in brain aluminium in Alzheimer's disease. However, McDermott et al (1979) later showed an age-related increase in aluminium in normal subjects and failed to show a statistically significant increase in patients with Alzheimer's disease compared with age-matched controls. Our own studies and recent data from Perl et al (1986) show clearly that considerable amounts of aluminium are plaque-related and tangle-related. Since plaques and tangles occur only in very small numbers in normal brain as opposed to the Alzheimer brain the whole pattern of distribution of these elements must be different. We need to know where aluminium and silicon are in the normal brain. Our imaging techniques so far haven't allowed us to study that.

Williams: What are the local concentrations of aluminium and silicon in parts per million?

Edwardson: The problem is that the plaque core is not homogeneous. In the central region levels of aluminium and silicon are very high, approaching 20%. We are sure there is very little protein there but we don't know what other carbon-containing compounds are present.

Wilson: Does the epidemiological evidence you cited suggest an association with water treatment rather than with the natural level of minerals in the water supply?

Edwardson: The increased standardized mortality rate (SMR) for dementia in Northumberland compared with Durham (Gardner et al 1984) is associated

with a considerable difference in the aluminium content of the water supplies. Levels added during treatment in Northumberland frequently exceed 0.2 p.p.m. whereas, in most of Durham, water supplies contain only natural aluminium and seldom exceed 0.05 p.p.m.

Williams: Could the aluminium:calcium ratio influence the effects?

Edwardson: That is very important.

Williams: Could natural, i.e. untreated, water contribute to those ratios?

Edwardson: It could indeed. The ratio of calcium and magnesium to aluminium in drinking water varies enormously between different regions in the two counties, almost to the extent of differences reported in the Guam studies. Some water comes from wells in Magnesian limestone and is very rich in calcium and magnesium while being low in aluminium. In contrast, some relatively soft river waters are heavily treated with aluminium. Also, fluoride levels have been reported to influence absorption of aluminium (Still & Kelley 1980) and there are large local variations in fluoride content.

Newman: In advanced cases of the acquired immune deficiency syndrome (AIDS) caused by HTLV-III there are symptoms of senile dementia. Do such patients show neuritic plaques?

Edwardson: There are no plaques in AIDS, to my knowledge. It is quite inappropriate to call those symptoms senile dementia. It is presumably virus-induced encephalopathy. The paired helical filaments which are typical of the tangles that occur in Alzheimer's disease and were reported in AIDS do not appear to occur in AIDS after all (Gibson 1985). But as AIDS becomes more widespread presumably sooner or later we will see co-existing pre-senile Alzheimer's disease and AIDS.

Birchall: What was the atomic ratio of aluminium to silicon, Professor Edwardson? Are the two elements separately deposited such that the centre of the plaque is rich in aluminium while silicon is outside? In which case it would be wrong to call the plaques aluminosilicates.

Edwardson: All of the plaques that we have now studied from senile and presenile cases of Alzheimer's disease and normal aged brains show coincident distribution of aluminium and silicon. Just occasionally we see a small area where one element occurs without the other. These spots tend to be in the periphery of the plaque core and their significance is unclear. We can't put a precise figure on the molar ratios. There is a lot of variability from plaque to plaque but a ratio of 1:1 is not unusual.

Birchall: What was the relationship between that ratio and the tangles and so forth? Did it need a high aluminium/low silicon content to get all the protein component disturbed?

Edwardson: We can't say anything about the relationship of this inorganic material to the protein component. Several groups have attempted to extract plaque cores by procedures which are so mild that the plaque core comes down with adhering cellular debris from the surrounding structures. It is so badly

contaminated that it is of questionable value in identifying specific plaque-associated proteins. In contrast, we strip off everything except the most insoluble protein, but we do not know how much protein is present in the plaque cores *in situ*.

Mann: Have any aluminosilicates or aluminium phases and silicon been found in plaque-type formations in other organs within the body, such as the spinal cord?

Edwardson: Not to my knowledge.

Richards: As you point out, stripping everything off is a very severe treatment. You remove much of the protein, the collagen and so on. Can you tell us anything about the amino acid composition of the little bit of protein that is left?

Edwardson: This is one of the major controversies in Alzheimer research at the moment. Some groups claim that it is a specific plaque core protein, not related to any other known protein. Other groups claim that the plaque core protein is related to the tangle protein. The consensus is that at the heart of this disorder lies an abnormal protein and what we are looking at is secondary mineralization. However, the coexistence of aluminosilicate and specific plaque proteins could equally well be explained as an attempt by the brain to isolate highly focalized deposits of aluminosilicate; perhaps glial cells secrete a protein which binds to the inorganic component, to isolate it. One can't conclude automatically that because the proteins are endogenous they got there first. This is a disease, after all.

Williams: The aluminium assault suffered by patients undergoing renal dialysis seems to support your view.

Edwardson: In dialysis dementia, the neuropathological changes observed in the brain are not those of Alzheimer's disease. There are not large numbers of senile plaques. However, an increased incidence of neurofibrillary tangles, correlating with tissue levels of aluminium in the same brain, has recently been reported (J. Kogeorgos, personal communication). That particular problem is still open for study.

Richards: The protein that is left must be very heavily cross-linked, otherwise you would take it out with all those treatments. Does anyone know the amino acid composition or the molecular mass of the material that is left after that severe treatment?

Edwardson: In 1983 Allsop et al reported on the amino-acid composition of plaque core protein and recently they have pointed out similarities in the composition of this material and perivascular amyloid protein (Kidd et al 1985). However, none of the groups working in this field have previously reported the presence of large amounts of inorganic material in isolated plaque cores—presumably because they have not looked for it.

Carlisle: Are the proteins that have been isolated from the neurofibrillary tangles quite different from those in the plaques?

Edwardson: Again, this is a controversial area. Kidd et al (1985) have also pointed out similarities between plaque core protein and paired helical filament protein found in tangles. However, immunocytochemical studies have indicated a heterogeneous protein content including both neurofilament proteins and antigens unique to paired helical filaments (Rasool et al 1984). The abnormality might be in the cross-linking rather than its amino acid sequence. It is now claimed that if the tangle protein is treated with different enzymes to modify the epitopes, increased staining with antibodies against neurofilament protein occurs.

Carlisle: We have undertaken a long-term study to determine the effect of diet on the uptake of silicon and aluminium in the brain of rats. We found that whereas the distribution of aluminium appeared to be rather constant over the brain, regional concentrations of silicon varied. For instance, in the hippocampus, caudate and lentiform nucleus silicon concentrations were greater than in the other nine regions examined. It appeared as if silicon had some function in the brain. Addition of aluminium to the diet reduced the silicon content significantly in those brain regions.

Edwardson: Are these bulk estimates?

Carlisle: Yes. It is important in nutrition to know the concentration of elements in the various brain regions as well as determining their localization.

Edwardson: In the Parkinsonism dementia of Guam and some other neurodegenerative disorders, extensive mineralization of cerebral blood vessel walls can occur. Thus there may well be a bulk increase in silicon but at sites that do not affect neuronal function. You said that the aorta was the organ with the greatest concentration of silicon. If the vascular bed can contain these high levels, how do you know it is not the vascular bed which is responsible for the differences between various tissues and organs?

Carlisle: The vascular bed of course would be included in the dissected brain tissue. Presumably, the vascular bed also contains relatively high levels of aluminium as well as silicon, as we find in the aorta, for example. However, aluminium content does not vary over the brain as is the case for silicon.

Williams: You were trying to concentrate on intracellular phenomena, weren't you?

*Edwardson:*No. We have drawn attention to the presence of aluminium as a common factor in both the senile plaque core and in the tangle-bearing neurons. These are the two major neuropathological features of Alzheimer's disease. The possible involvement of this toxic element in the pathogenesis of this disorder must be considered.

Daniele: Have you investigated the effect of aluminium silicates on neuronal cells in tissue culture, particularly with regard to their effect on certain enzyme systems such as acetylcholinesterase?

Edwardson: We are doing that at the moment. Previous studies with aluminium compounds *in vitro* haven't produced many interesting effects. However,

even in the most severe cases of Alzheimer's disease only selected subpopula-
tions of neurons appear to be affected. There are severe cognitive and emotion-
al deficits but most of the nervous system is still working. Most studies involving
tissue culture have not used the cells affected in Alzheimer's disease, e.g.
pyramidal cells from the cortex or magnocellular neurons from the nucleus
basalis, so perhaps it is not surprising. Recently we have put washed isolated
plaque cores into tissue culture. A problem we hadn't expected is that the
cholinergic neuroblastoma–glioma hybrid cells just refuse to grow around the
isolated plaque cores.

Werner: How many patients in the north-east of England are your observa-
tions based on? And with what levels of aluminium in the drinking water were
those numbers finally correlated?

Edwardson: We have not made any correlations yet. We are looking at the
content of aluminium, magnesium, calcium and fluoride now.

The numbers of cases varied. Dementia is the most prevalent disorder and in
the 10-year period 700–800 cases were reported on death certificates. There
were about half this number of cases of motor neuron disease.

People have previously asked whether there could be any relationship be-
tween these disorders and aluminium in the drinking water. The standard reply
has always been that even when very large levels of aluminium are put in during
treatment this provides only a small proportion of the total dietary intake of
aluminium. However, we do not know what proportion of total dietary alumi-
nium can be absorbed under conditions of low calcium intake. According to
figures given by the Ministry of Agriculture, 60% of the aluminium in the
typical British diet is in cereal and vegetable matter. What form is this alumi-
nium in? I suspect that it is probably there in insoluble forms.

Williams: Phenolate compounds and complexes are very common in the
plant world but not so common otherwise. The phenolate complexes of silicon
which we discussed earlier (p 105) can be made with aluminium too. Such
complex formation is a very good way of absorbing aluminium. Soil can lock up
a lot of aluminium in phenolate systems. If such compounds are taken up in
biology and then metabolized they would presumably go straight out again. But
the aluminium that is put in the water is basically the hydroxide, which reverts
to free aluminium as soon as it comes into somewhat acidic regions—say of the
body. $Al(OH)_3$ reverts rather more easily than aluminate-phenolate com-
plexes. There is no direct answer to the question but the chemistry suggests that
aluminium hydroxide is likely to be a more risky chemical than aluminium
phenolate, simply because it becomes soluble more easily and gives free
aluminium.

Edwardson: So the added load in water treatment may form a significant
proportion of the potentially absorbable load. This is a key issue and it is
surprising that it has not been answered.

Williams: We need a lot more clear-cut chemistry. Certain organic acids form

quite strong complexes with aluminium at pH 4 (Tam & Williams 1986) but by that pH level aluminium hydroxide is dissolving very easily. The hydroxide would not compete with oxalate for Al(III) very effectively around pH 4 or 5. At pH 7–11 aluminium hydroxide will form. The exact chemicals present govern the state and availability of Al(III), as does the pH. This is where the problem of calcium competition, the calcium–aluminium balance, also comes in. Aluminium has a higher dependence on acidity than calcium and is bound strongly at pH 7. As the pH is lowered, becoming more acid, the free aluminium concentration is dependent on a higher power of the hydrogen ion concentration than the free calcium. Aluminium is released from some sites and becomes a stronger competitor for some calcium sites as the system becomes more acidic. That is one of the problems with acid rain, I would say.

Espie: Alfrey (1985) has reported that normal, daily consumption of aluminium from food is between 2 and 5 mg.

Werner: A figure for aluminium in plants is about 0.01% (dry weight) in spinach leaves and 0.007% in potatoes. Eating 200 g dry weight of this food per day would mean an intake of 20–25 mg of aluminium.

Farmer: Did you say there was an analogy between the core material and the synthetic aluminosilicate gels that I spoke about earlier, Professor Edwardson?

Edwardson: I didn't describe it as an analogy but as a red herring. However, it does show that we cannot rely on the specificity of any of the empirical neuropathological stains to identify this material; because something is fibrous and stains with Congo Red doesn't mean it is protein. However, I am sure that the plaque core material is not imogolite!

Farmer: The differences between brain plaque aluminosilicate and synthetic materials are interesting. The aluminium to silicon molar ratio of the plaque material seems near 1:1, whereas that of imogolite is 2:1, and it won't pick up any more silicon. But if we make an aluminium silicate precipitate at neutral or alkaline pH the ratio approaches 1:1. Some of the aluminium is then incorporated in a tetrahedral aluminosilicate network.

Another question was whether the plaque material was a pure aluminium silicate gel or whether it was in an organic matrix. The 20% of aluminium and silica *in situ* that you quoted is the highest possible figure for an anhydrous aluminium silicate gel. For example, imogolite dried at 100 °C contains 13% silicon and 25% aluminium. An electron probe can, however, dehydroxylate such gels, and so increase their metal oxide content.

Edwardson: That is at the centre of the core, not distributed throughout the core. The aluminium and silicon is right in the middle of the bulky particles we see by scanning electron microscopy and we don't know what the rest of that material is. Using X-ray microprobe analysis we can only measure the inorganic components and we have no value for the carbon and so on. With solid-phase NMR on larger samples we should be able to answer this question soon.

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Effects of silica on lung collagen

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Abstract. A single intratracheal injection of 50 mg crystalline silica (quartz) into rats produces silicosis. This animal model may be used to study collagen metabolism during the early, middle, and late phases of lung injury, corresponding respectively to the stages of lung injury, development of discrete granulomas, and development of mature silicotic nodules. The early phase is characterized by a rapid increase in the rate of synthesis of lung collagen (within one week of instillation) and increased deposition of excess lung collagen (significant increases within two weeks of instillation). Later phases are characterized by a continuing increase in deposition of excess lung collagen for at least one year after instillation. Silica-induced fibrosis is unique among all the animal models (and in most human fibrotic diseases) thus far examined, in that the excess collagen deposited in the lung contains normal ratios of the two major collagen types of the lung: types I and III. This collagen is nonetheless biochemically different from normal lung collagen. There are reproducible and characteristic differences in the intermolecular cross-links of the collagen in lungs from rats injected with silica. Within one month of silica instillation (the earliest time point examined thus far), an increased hydroxylysine content of collagen can be appreciated. The reducible difunctional cross-links are also more likely to be derived from hydroxylysine (i.e. the ratio of dihydroxylated to monohydroxylated cross-links increases). Within four months of silica instillation (and increasingly thereafter), increased amounts of the mature trifunctional cross-link hydroxypyridinium (derived from three residues of hydroxylysine) can also be appreciated, seemingly paralleling the evolution of mature silicotic nodules in these lungs. These changes in cross-linking of lung collagen seem to be common to all the animal models of pulmonary fibrosis examined, and are also consistent with changes occurring in human fibrotic lungs. Preliminary observations suggest that the locus of cross-linking remains the same: hydroxylysine replaces lysine in the primary structure of a specific collagen α chain to form the altered cross-links. Thus, there may be molecular markers for the collagen of fibrosis in diseased lungs.

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Silica-induced fibrosis of the lungs is a disease known since antiquity. Its association with dust inhalation in mining and quarrying was recognized by Agricola in the Middle Ages. Silicosis has remained the prototypical occupational disease for several centuries. There is a greatly increased risk of

tuberculosis in individuals with silicosis. Before the advent of effective che-
motherapy for tuberculosis, this was a deadly combination (and still may be in
many underdeveloped countries). At present, at least in the United States and
the United Kingdom, silicosis is more of medicolegal concern for compensation
of affected individuals. Very recently, concerns about increased risks of lung
cancer in workers exposed to crystalline silica have been raised, based on
epidemiological studies and animal research. Laboratory animals with ex-
perimentally induced silicosis have been studied for at least 40 years and
remain an important focus of work in lung disorders, biochemistry and cell
biology. In this paper we will mainly discuss the results and implications of the
more recent animal studies, with emphasis on our own work. Earlier work in
this area has been thoroughly reviewed in an authoritative monograph by Zaidi
(1969), and more recently by ourselves (Reiser & Last 1979).

Experimental methodology

Silicosis can be induced in animals that either inhale crystalline silica, prefer-
ably quartz of an optimal size, or receive a direct intratracheal injection of an
aqueous suspension. Results with either protocol seem to be dose-dependent,
so the total burden of silica in lung is an important variable to consider when
various experiments are compared. As much as 50 mg may be intratracheally
instilled as a single bolus. As a practical matter, the highest concentration
achievable in inhalation chambers is probably about 10–20 mg/m^3 (i.e. 10–20
µg/l). The maximum dose to which a rat with a minute volume of about 100 ml
can be exposed per eight-hour day is therefore less than 1 mg/day. After
correction for losses in the nose, five-day work weeks, etc., it is clear that
inhalation studies, even chronic ones, usually involve much lower dose rates
and generally lower total lung burdens than do studies using intratracheal
injection. Since we know little of the implications of acutely overwhelming the
particulate clearance mechanisms of the lung compared with those of con-
tinuously challenging these defences over time, interpretation of results from
experiments using intratracheal injection may be an area of some controversy
for those concerned with questions of human health. On the other hand,
intratracheal injection offers a convenient and inexpensive technique for
descriptive and mechanistic studies. In our work we have chosen to use the
intratracheal route as a standard procedure.

   Another major consideration in animal research is the time frame of a given
experiment: acute, sub-chronic, or chronic. Intimately related to duration of
the experiment are questions of the total dose of silica to the lung and of the
appropriate assays to quantify the effects. We have shown significant responses
of the lung to silica as early as one week after intratracheal injection of a single
dose of 50 mg quartz (Reiser et al 1982). On the other hand, the full spectrum of
the lung's response to silica may take 6–12 months to manifest itself after a

single 50 mg dose is instilled (Reiser et al 1983), with correspondingly lesser responses if lower doses are instilled or inhaled.

A third important consideration is the animal species and the specific fibrogenic agent (and, for silica, the specific chemical form, e.g. quartz versus amorphous) chosen for study.

Collagen metabolism in silicotic lungs

Use of biochemical methods for examining the collagen of fibrotic lungs seems a logical approach to understanding the underlying pathogenic mechanisms in silicosis. The simplest question to ask from an analytical point of view is whether there is more collagen in a silicotic than a normal lung. The answer is yes: there may be as much as four times the amount as in age-matched controls (Reiser et al 1983). There seems to be a dose–response relationship between amount of quartz instilled and amount of collagen (hydroxyproline) deposited in the lung (Swensson 1971). There is also a time–response relationship (Swensson 1971), such that it may take several months to appreciate the increased collagen content of rat lungs after instillation of relatively low doses of crystalline silica (i.e. ≤ 10 mg instilled). However, at higher doses of silica such an increase in lung collagen can be appreciated quite rapidly, as early as 1–2 weeks after instillation of 50 mg in our studies (Reiser et al 1982). It is doubtful that changes as dramatic as a threefold increase in collagen content could actually occur after only six days, as Chvapil et al (1979) reported, but a change of this magnitude occurs after several months. In this respect, silicosis does not differ from other animal models of experimental pulmonary fibrosis. For example, intratracheally instilled bleomycin causes an increase in rat lung collagen that can be appreciated two to three weeks after instillation (Hesterberg et al 1981). The important difference between silica and bleomycin, ozone, butylated hydroxytoluene and other pneumotoxicants seems to be in the continuing deposition (for at least a year after instillation) of excess collagen in lungs of animals in the silica model. The other agents all seem to cause a rapid increase in lung collagen to a plateau value which is usually 0.2–0.5–fold higher than that in age-matched controls. Such increased levels of lung collagen do not seem to persist indefinitely (Haschek et al 1982). However, there are also other animal models available of so-called 'progressive fibrosis', usually involving insult with two agents (e.g. bleomycin plus 70% oxygen, butylated hydroxytoluene plus 70% oxygen [this model is specific for mice among animals tested], bleomycin plus various immunosuppresive agents) in a carefully timed sequence. These models also result in persistent fibrosis, with maintenance of an increased content of hydroxyproline in lung (Haschek et al 1982). However, these models seem to reach a peak accumulation of hydroxyproline within weeks of insult, in contrast to the continuing increase over one year observed with silica.

Such observations of increased lung collagen in silicosis quite naturally give rise to our next question, 'is the excess collagen deposited in the silicotic lung different from normal collagen'? The answer is clearly yes, but it took us a long time to find this out, and therein lies another important difference between silica and other fibrogenic agents. Our initial experiments with silica as a fibrogenic agent were based on our naive (and, it turned out, erroneous) expectation that it might give rise to a slow, progressive fibrosis that in some respects would act as a model for chronic pulmonary fibroses in humans (sarcoidosis, idiopathic pulmonary fibrosis [IPF]). The pioneering studies of Kang and co-workers (Seyer et al 1976) showed that normal lung in humans contains about a 2:1 molar ratio of type I to type III collagens, and that these two types account for most (>90%) of the total lung collagen present. These workers examined the ratios of collagen types in lungs obtained *post mortem* from patients dying of IPF and found a shift towards an increase in type I collagen, with a new ratio of about 4:1, on average (Seyer et al 1976). We had demonstrated that similar shifts take place in the pool of newly synthesized collagen in animals with lungs acutely damaged by fibrogenic agents such as bleomycin, ozone and paraquat (Reiser & Last 1981). Therefore, it seemed logical to perform similiar analyses in lungs from rats exposed to silica. To our surprise, despite the increases in collagen synthesis rate and the greatly increased collagen content of the silicotic lungs, we found that collagen was synthesized and deposited in the normal ratio of types I:III, 2:1 (Reiser et al 1982, 1983) from one week to one year after instillation of the silica. In this respect, silicotic fibrosis is unique among the animal models of lung fibrosis we have examined (Reiser & Last 1981, Haschek et al 1982), and it is also different from the situation in acutely (Last et al 1983, Shoemaker et al 1984) and chronically (Seyer et al 1976) fibrotic human lungs. At present, we have no explanation for this difference between silica and other fibrogenic agents. We find it a fascinating reason for continuing to study silicosis, in that we would very much like to understand the underlying biochemical and cellular mechanisms whereby the silicotic lung is able to mount its unique response while laying down large amounts of collagen.

In our most recent studies we have examined another important property of lung collagen, its higher-order structure as determined by the ability of collagen chains to become cross-linked to one another by specific, lysine-derived, covalent bonds. In this process, specific lysine or hydroxylysine residues are oxidized to their corresponding aldehydes, which can then react with each other or, more importantly, with ε-NH_2 groups of lysine or hydroxylysine residues (via a Schiff-base intermediate) to form difunctional (and trifunctional or tetrafunctional) cross-links. We have speculated elsewhere (Last 1985a) that there are good theoretical reasons for assuming that there might be molecular markers of 'fibrotic collagen' that differed from 'normal collagen', such that the lung might modulate the ultimate metabolic fate of

these collagen pools in different ways. Such speculations led us into a systematic examination of collagen cross-linking in normal rat and mouse lungs and in lungs of rodents with experimentally induced fibrosis. To do this, we exploited new techniques of HPLC on reversed-phase columns that we developed for this purpose (Reiser & Last 1983).

Since tissue from many of the same lungs that we had examined biochemically and histologically in our earlier studies remained in the freezer, we began our experiments with these silicotic lungs. We made two observations that are directly relevant here. When we reduced lung tissue with NaB^3H_4 to stabilize and label the difunctional cross-links of interest, we found the ratio of dihydroxylysinonorleucine (DHLNL) to hydroxylysinonorleucine (HLNL) in normal lung tissue to be about 3.4:1 for rats from one to nine months after silica instillation. The DHLNL:HLNL ratio for the silicotic lungs was about 8:1 at one and four months after instillation, and about 11:1 for the six-month and nine-month samples. These changes are consistent with an increase of about 50% in the hydroxylation of lysine to hydroxylysine in the collagen of these silicotic lungs (Reiser & Last, unpublished work). The second observation we made concerned the non-reducible ('mature') trifunctional cross-link hydroxypyridinium (OHP), derived from the condensation of three residues of hydroxylysine. Not surprisingly, OHP content of the collagen in lungs of the silicotic rats gradually increased over the duration of the experiment, compared with age-matched controls, such that at nine months after silica instillation the lungs contained about twice the normal lung content of OHP. Thus, we identified the following changes in 'silicotic collagen': (1) higher content of hydroxylysine, (2) greater ratio of DHLNL:HLNL, (3) higher content of OHP. The first two changes were appreciable by one month after silica instillation (the first time point we studied), while the third became apparent four months after silica instillation.

We have had the opportunity to do similar studies on a limited basis in rats and mice receiving intratracheally instilled bleomycin, in mice receiving bleomycin plus oxygen, and in a small number of lungs from infants dying of respiratory distress syndrome of the newborn, a (pre)fibrotic lung disorder. Our results are consistent with those from the silica model: a rapid shift in DHLNL:HLNL ratio and a long-term increase in OHP content where animals survived long enough for this change to manifest itself. In this sense, then, the silica model is completely consistent, as far as we can determine, with our other animal models of fibrosis. More important, perhaps, is our observation that 'normal' and 'fibrotic' lung collagen are different, and that DHLNL:HLNL ratio or OHP content, or both, are molecular markers for 'fibrotic' collagen.

We have also done preliminary experiments in a different tissue that are relevant to these observations. We examined collagen cross-links in the urine of rats for up to one week after intratracheal administration of silica. Although the rationale for studying collagen breakdown where excess collagen is depo-

sited is somewhat obscure, the potential application of an assay for a urinary metabolite in clinical medicine seemed to us to justify this approach. To do these experiments, we placed rats in metabolic cages after instilling silica or saline (control animals) and collected urine in 24-hour batches from each animal. Individual or pooled samples were treated with 70% ethanol to precipitate and concentrate the peptides of interest. The resulting precipitate was hydrolysed with acid and analysed by HPLC.

Two observations from these experiments appear interesting. First, silicotic rats appear to excrete a much lower amount of reducible difunctional cross-link (DHLNL plus HLNL) than do control animals during the first week after silica administration. Since we do not know the relative contributions of different tissues (especially bone) to the total excreted pool of difunctional cross-links, we cannot yet interpret these findings. However, they are tantalizing in that we are observing a reproducible difference between the silica-instilled and control animals. Second, using the technique described above, we have been unable to detect OHP in the urine of either control or silica-instilled rats. The equivalent of about half the total urine collected from a single rat for 24 hours has been examined by a sensitive fluorometric assay. Since we can readily detect OHP in human urine when we use this protocol, we are seeing a species difference, or an age-related difference (the rats are two to three months old in these studies), or a problem in the sensitivity of detection. Further studies using additional concentration and purification steps are necessary before we can properly interpret these findings.

We are also actively investigating the location of the cross-linking sites on the constituent chains of lung collagen that are responsible for the shift in DHLNL:HLNL ratio we observe in 'fibrotic collagen'. For example, we have identified a cross-linked peptide containing HLNL in cyanogen bromide digests of reduced collagen from rat tail tendon. This particular peptide seems to be a cross-linked intermolecular species containing α_1(I)CB–7 and α_2(I)CB–1. The comparable cross-linked peptide can be isolated from CNBr digests of reduced whole lung tissue. This peptide contains HLNL in control animals (normal collagen) and DHLNL in rats examined six months after instillation of silica (fibrotic collagen). Preliminary observations suggest that the same residues are involved in the cross-link; however, the lysine of α_2(I)CB–1 seems to be hydroxylated to hydroxylysine in 'fibrotic collagen'. This observation raises fascinating questions about the biochemical basis of pulmonary fibrosis. For example, is the excess hydroxylation of collagen (and its cross-links) that we are observing related to increased levels of lysyl hydroxylase in damaged lungs? We know that the activity of lysyl hydroxylase is indeed increased in many animal models of pulmonary fibrosis (Last 1985b). On the other hand, the extent of hydroxylation of lysine residues in collagen is also thought to be controlled by the rate of folding of chains while they are being synthesized. The rate of synthesis of collagen chains may also differ among different types or

clones of cells, and may be a factor in defining 'fibrotic collagen'. These kinds of questions seem to be approachable experimentally with modern techniques of biochemistry and cell biology.

The similarities and differences between silicosis and other models of experimental lung fibrosis in silicosis have continued to fascinate us. Key questions remain unanswered that may be further examined by such comparative studies. For example, if fibroblasts make 'fibrotic collagen', as most workers in this field assume, how are silicosis fibroblasts able to make 'fibrotic collagen' without at the same time shifting the ratio of collagen types being produced? Is the ability to make 'fibrotic collagen' a property of certain cell clones or is it under the control of factors produced by other lung cells, as suggested by several workers? Is 'fibrotic collagen' different enough from 'normal collagen' to be a rational target for therapy of these lung diseases that are at present incurable? These are areas of research that should keep workers in the field occupied for many years to come

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DISCUSSION

Williams: As far as you know, these effects are due to SiO_2 in one form or another. Is it known whether other insoluble particles of a similar critical shape do the same thing?

Last: When we examine the capacity of insoluble particles to induce pulmonary fibrosis, as crystalline quartz does, aluminium and amorphous silica are inert and do not produce these types of changes. Asbestos has some similarities in inducing asbestosis and fibrosis but it does not do this on the same scale as silica. Glass does not do this, nor does talc.

Williams: We need now to get the biochemistry clear. There is an endoplasmic reticulum space, a cytoplasm space, and the external space where the collagen is placed in final form. Hydroxylation occurs on or before the protein passes through the membrane and before glycosylation. The hydroxylation of lysines is really either on the membrane or in the cytoplasm and the silica particles must reach this region of the cell to cause an effect there.

Last: The enzymes are membrane-bound.

Williams: They are iron enzymes. Cross-linking occurs extracellularly through copper-dependent proteins.

Last: In terms of the hydroxylation of proline, this issue is probably irrelevant. The level of hydroxylation of proline has something to say about whether or not a triple helix is formed but it says little about fibre structure. The hydroxylation of lysine, which is important for fibre structure and is apparently changing in fibrosis, is an intracellular event.

Williams: So that is the major event, and the cross-linking is secondary?

Last: That is a peculiar point of view, but yes.

Williams: We have to think about how silica affects a process in the cell in the first instance.

Last: In all models of fibrosis that have been studied, total lysyl hydroxylase increases in the lung. However there are observations that say such an increase will not necessarily cause inappropriate hydroxylation. If the chains are made more slowly so that they don't form their folded structure as rapidly, then additional hydroxylation of lysine residues may occur. The implication is that, whether the cells that make it are different or the process is different, the chains are folding more slowly.

Sullivan: Is the lysyl hydroxylase activity or the number of enzyme molecules changed in this response?

Last: The only thing that has been measured is total activity. I don't know the answer.

Richards: Perhaps the cross-linking of collagen alone is not enough to explain fibrosis. Cross-linking between different proteins such as fibrin, fibrinogen and other matrix proteins and collagen may be more important in the whole fibrotic process. That would change the idea that the cross-link with collagen is the most important. As you pointed out, silicosis usually occurs with calcification and there are very good enzymes that need calcium to activate them. Transglutaminase, for example, loves to cross-link all these different proteins.

Last: We don't have the tools for dealing with those questions yet.

Wilson: You said that glass fibre doesn't induce fibrosis, but isn't there evidence that, in the correct aspect ratio, it does?

Last: Most of the data I have seen with fibreglass say that if an overwhelming amount of this material is used, the characteristic walled-off bodies are seen in the lung but there is a very localized response, not the usual type of fibrosis. Presumably a granuloma is forming or there is some sort of walling off occurring. I don't know of any ordinary fibrosis caused by fibreglass.

Daniele: A recent report indicates that glass fibres can cause fibrosis, especially when their size is similar to crocidolite asbestos dusts (Goldstein et al 1983).

Wilson: And progressive fibrosis too. I assumed it was because in this mass that you are administering there was not a sufficient amount of fibres with the critical ratio present to cause the problem.

Dobbie: What is known about the surface chemistry of quartz crystals?

Hench: Later I'll be showing that quartz crystal surfaces, under certain conditions, exert very strong epitaxial effects on the polymerization of simple amino acids. But you can change the surface conditions of the quartz and the epitaxy is lost. It would be interesting to know whether anybody has done experiments on quartz that has undergone surface treatments that eliminate silane bonds and protein epitaxy.

Werner: In the 1950s A. Weiss and co-workers found an iron phosphatosilicate in silicosis. Did you find similar products that might be specific to silicosis in the tissue?

Last: The experimental models of deliberately induced silicosis have tended to use exceedingly pure silica, which seems to be sufficient to provoke silicosis. I don't know of any other pure compound that provokes the same type of response. Clearly this is not the same as the silicosis in humans, where the dust is very complex in terms of characterizing the potential interactions of agents.

Werner: The point was that in the lung this iron phosphato-silicate was only there when silicosis developed. It wasn't put in, but it developed in the tissue.

Last: We didn't try to examine that. These animals are exceedingly clean-lunged and have not been exposed to any kinds of dusts or foreign agents, so it is unlikely they would have any casual foreign bodies in the lung. There would have to be a deliberate synthesis from body stores if something like that occurs.

Daniele: We have also developed an experimental model of silicosis in the guinea-pig, using the intratracheal instillation of quartz particles (Dauber et al 1980, Daniele 1986). The histopathological features are similar to what has been described in the rat by Dr Last. Also, collagen synthesis and content in the lungs of the experimental animals were increased but the ratio of total collagen to lung weight and the ratio of type I to type III collagens were relatively constant. In this experimental model, we have also examined the possibility that an increase in fibroblast number might explain the increase in collagen content. Alveolar macrophages that were recovered from experimental animals six months after the instillation of silica liberated a factor that stimulated lung fibroblast proliferation (Lugano et al 1984). The exact identity of this factor and its relationship to interleukin 1 are unsettled.

Our studies have also focused on the role of the alveolar macrophage in modulating the early inflammatory response. These studies involved both *in vivo* and *in vitro* exposure of alveolar macrophages to silica particles (Lugano et al 1981, 1982). For example, shortly (weeks) after the intratracheal instillation of silica particles, alveolar macrophages recovered from experimental animals liberated a factor(s) that was chemotactic for neutrophils, providing one explanation for the early recruitment of neutrophils in this model of experimental silicosis.

We have also been studying the impact of silica on certain host defence functions of alveolar macrophages (Daniele 1986, Dauber et al 1981). It is recognized, for example, that the alveolar macrophage is the principal defender of the distal airways in removing and disposing of inhaled particles and microbes. We examined the effect of silica on the functional activity of certain receptors and enzyme systems that are located near or at the plasma membrane and are involved in the microbicidal and defensive functions of the alveolar macrophage. Alveolar macrophages were recovered from experimental animals that had been exposed to silica (quartz particles) six months previously as well as after short-term *in vitro* exposure (2 h) to sublethal concentrations of silica; the latter included doses of silica particles that did not kill alveolar macrophages after short-term culture (24 h). In both situations, alveolar mac-

rophages had an impaired ability to recognize particles that were opsonized with IgG (Fc receptor activity) and an impaired formyl peptide receptor that is important in signalling cell motility and in the activation of oxidase enzyme systems responsible for the generation of oxygen radicals. At this stage, it is unclear where is the locus of silica-induced injury. The injured structures could include the receptors or their associated enzyme systems (oxidases) or the subcellular structures (cytoskeletal elements) that are involved in cell motility. A more immediate question is what is the unique biochemical property of quartz particles, as compared to glass or silicates, that allows them to interact with the cell membrane and cause injury? One hypothesis is that silica induces lipid peroxidation of the plasma membrane, resulting in injury to membrane components.

Dobbie: As I shall show later, cell membranes can be exposed to dissolved silica for a long time.

Volcani: In what way does your system differ from Heppleston's (1978) with regard to the complexity of the effect of silica on collagen production, Dr Last?

Wilson: The collagen-stimulating factor identified by Heppleston and Styles sounds like an impure version of what you are trying to identify.

Last: I assume you are talking about the theory that macrophages are able to release a factor that directly stimulates enhanced collagen synthesis by fibroblasts and thereby causes fibrosis. That work has not been reproduced by anyone else, to my knowledge, despite many attempts. The kinds of assays being used were essentially looking at increases in hydroxyproline in tissue culture plates. Perhaps these were not well controlled either for increases in fibroblast number or for other effects such as stimulation by the culture medium or whatever is added with the material. Most people, except Professor Kulonen's group in Finland, would assume that interactions between macrophages and fibroblasts would be mediated by factors that cause the fibroblasts to be either recruited or to proliferate, as opposed to directly stimulating collagen synthesis by fibroblasts. Kulonen's group (Aalto et al 1980) isolated and partially purified a factor that they thought was directly stimulating collagen production by fibroblasts that seemed to co-purify with, but be different from, ribonuclease. Given the strongly basic character of ribonuclease, it is not too amazing that something might be co-purified with it. That work is very difficult to interpret but there are a lot of papers and a lot of people have been involved. There is still a possibility that there is a direct fibrogenic factor. The simple hypothesis that a macrophage gets angry with the silica, opens up and puts out a factor that stimulates more collagen is too simple and probably is not correct.

Richards: There is a tremendous change in the epithelial surface in the silica-treated lung. Changes in the epithelial surface produce very active growth factors which stimulate fibroblasts. The changes in the epithelium are more important to fibroblasts because epithelial growth factor is possibly a more potent mitogen than macrophage growth factor. Even then you don't

need any of those mitogens at all, because if you just put silica with fibroblasts they will grow faster, and when they grow faster they lay down more collagen; thus none of the factors may be necessary if silica dust penetrates to the interstitium of the lung (Richards & Curtis 1984).

Williams: Therefore we should not just try to explain protein production but protein modification. That seems to me to require an explanation whereby the silica in some way can interact perhaps with the cell membrane. This is not quite the same as getting inside the cell.

Daniele: The issue of how the silica interacts with biological membranes seems crucial.

Last: That may be a red herring. You may be selecting different clones of fibroblasts if you go back to the fibrotic lung.

Volcani: Drs Patricia Burnham and Walter Desmond in my laboratory (unpublished) have done extensive studies on the effect of monosilicic acid and polysilicic acid on cells in tissue cultures. We wanted to see if the 'solubility theory' (see Parks 1974) which prevailed about 20 years ago could help us to understand silicosis. Why does it sometimes take so many years for the disease to manifest itself? The idea was that there was probably a leaching of silicic acid from quartz, which takes time. Solubilization is localized, consequently it is the silicic acid that is the culprit in that disease. That was what the theory said. Is it correct?

Using two types of cells (rat lung fibroblast cells and cat lung epithelial, i.e. type II, cells), we determined the effects of monosilicic acid and polysilicic acid on their growth. We also studied the cellular changes by electron microscopy. Monosilicic acid did not affect growth but the polysilicic acid caused extensive vacuolization of all cells and the dramatic internalization of the plasma membrane of these cells into the lumen, forming vacuoles that digested the internalized plasma membrane. In some cases the cells recovered completely, in other cases they died.

The second dramatic observation was that polymeric silicic acid in the presence of monomeric silicic acid produced the binucleated and tetranucleated cells. In other words this mixture of silicic acids deterred cytokinesis and consequently resulted in the appearance of polynucleated cells which gradually degenerated. We found that in the pre-polysilicic acid-treated cells the monosilicic acid resides on the surface of the plasma membrane.

We have no good explanation for the formation of the polynucleated cells or for internalization of the plasma membrane other than the possibility that these concentrations affect microfilaments or microtubules and thus affect the cytoskeletal system of the cells. That is hypothetical and we have no experimental evidence for it.

Last: Does that imply that amorphous silica should be more fibrogenic than crystalline silica?

Volcani: Not necessarily. One of the problems is that we used fairly high

concentrations. This is a very interesting system for producing tetranucleated cells. It is also exciting to have a very effective system for internalization which enables one to study plasma membrane turnover.

Williams: Enzymes are localized in particular membrane regions and their properties may respond to changes of shape of the membrane. If the specificity is associated with membrane shape and the membrane takes up a shape which is not natural to it, through interaction with a particle, then the proteins will probably redistribute themselves. The shape of a membrane reflects the composition of that membrane. Most of these membrane enzymes are in some way organized relative to one another and their types of activity may alter because of changes of organization after changes in membrane shape.

Last: It seems that we are getting two diametrically opposed schools of thought here. One says that the soluble silica can move around, while the other says that the insoluble silica has a defined shape all the time. Is there any way of reconciling these two?

Williams: Only by your experiments!

Birchall: In work in my group we heated up precipitated amorphous silica to various temperatures for various times and then looked at what it was like. Eventually X-ray lines pop up and you can characterize it. This material was fed to a macrophage culture. What is measured is membrane damage and the results were quite clear. Amorphous silica had some small effect on membranes which increased as the silica surface dehydrated. When the silica began to show a few lines in the X-ray, that damage began to get progressively worse.

Wilson: Was the damage connected with negative charges on the surface?

Birchall: Not that we could find. It is known that the surface can be protected in one or two ways so that the silica is inert. One is with polyvinylpyridine-1-oxide and the other one is by treatment with alumina.

Richards: The point to remember is that the membrane probably never sees silicon dioxide in a biological system. It sees silicon dioxide plus a biological coat. In interactions between minerals (coated) and the cell, biological material of the mineral coat is interacting with biological material of the cell membrane.

Birchall: Yes, but the damage is done on the way out when the coat has been removed, not on the way in.

Richards: Every mineral probably has its own biological absorption pattern (Desai & Richards 1978).

Williams: And it may also have its own shape because the biological coat has its own shape.

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Urinary and serum silicon in normal and uraemic individuals

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Abstract. The urinary excretion of silicon (Si) in humans was studied in normal subjects on a low Si diet, a normal diet, and after ingestion of silicate antacid. Measurements of 24–hour urinary excretion of Si showed that urinary Si was derived mainly from dietary intake. The serum concentration of Si was determined in normal individuals and in patients with chronic renal failure. In health, serum Si is maintained within a narrow range, but a significant hypersilicaemia occurs in uraemia. The concentration of Si was measured in the water supply, dialysate and pre-dialysis and post-dialysis serum in patients on regular haemodialysis in three areas with low, intermediate and high concentrations of Si in the water supply. Si was removed during dialysis in the region where it was naturally low in the water or where reverse osmosis was used, but it was dialysed into patients in regions with intermediate and high concentrations in the water. Serum Si levels returned to normal after renal transplantation. Preliminary analysis of the geographical variation in the Si content of tap water suggests that uraemic hypersilicaemia may protect haemodialysed patients from the development of aluminium dementia. The kidney would appear from these studies to be the major organ for elimination of absorbed Si.

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Our initial interest in silicon was stimulated by a paper on the possible causal association between the silicon content of drinking water and a chronic interstitial nephritis known as endemic or Balkan nephropathy (Marcovic & Arambasic 1971). This disorder, which progresses slowly but inevitably to renal failure, is found only in specific locations in Yugoslavia, Bulgaria and Romania. Marcovic & Arambasic (1971) had succeeded in inducing lesions similar to those in endemic nephropathy by adding a suspension of finely ground quartz from one of the endemic regions (Kolubara) to the drinking water of guinea-pigs for a period of six months. They therefore advanced the hypothesis that endemic nephropathy might be due to the diffusion of silicate materials from eroded acid granites into the local drinking water.

Since these geological features are by no means unique to the regions of

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endemic nephropathy it would not be unreasonable to expect even sporadic cases of chronic interstitial nephropathy in areas of similar geomorphology. We therefore repeated Marcovic & Arambasic's study, substituting pulverized Scottish rocks (quartz, granite). Magnesium trisilicate BP, a synthetic therapeutic silicate antacid, was also tested. In this study (Dobbie & Smith 1982a), focal tubulo-interstitial nephritis, affecting mainly the distal nephron, was induced in guinea-pigs receiving a suspension of Scottish quartz or magnesium trisilicate but not in those receiving Scottish granite. In a geographical survey of the silicon content of drinking water in 28 locations in Great Britain, reported in the same paper, we showed that the concentration of soluble silicon was highest in the south and east, in hard water, non-granite areas. Conversely, the lowest concentrations of silicon were encountered in the north and west, areas of soft water where granite rocks are widespread. Although these studies tended to deflect interest from a possible causal relationship between granite and sporadic or endemic nephropathy, they reinforced a long-held suspicion that silicates are nephrotoxic.

Subsequent studies using atomic absorption spectroscopy showed that in humans the concentration of silicon in body fluids (bile, pancreatic juice, amniotic, cerebrospinal, pleural, ascitic and synovial fluids) was similar to that found in serum but considerably inferior to that found in urine (Dobbie & Smith 1982b). These findings suggested that the kidney might be the main or only organ engaged in the elimination of absorbed silicates in humans.

It thus became increasingly apparent from these early studies that a comprehensive investigation of the role of the kidney in silicon metabolism, excretion and toxicology was long overdue. We therefore embarked on a series of investigations of the renal handling of silicon in humans, in health and in the uraemic state.

Measurement of Si in biological fluids

After oxygen, Si is the second most abundant element in the environment and is found as an impurity in most materials. The first chemical methods used to determine Si were fraught with the problems of contamination and phosphate interference. With the advent of plastic laboratory ware Si contamination was minimized. Large quantities of phosphate present in some biological fluids, particularly urine, were difficult to eliminate in the analysis of Si by the molybdenum blue method (King et al 1955). More recently Jankowiak & LeVier (1971) have described precipitation methods for the effective removal of phosphate in biological fluids and tissue.

Flame atomic absorption spectroscopy

We have had favourable experiences with using flame atomic absorption

spectroscopy (AAS) for analysing Si in biological samples. Sample treatment, precision and viscosity were studied. We also compared this method with the colorimetric method and measured the recovery of Si added to serum.

A Perkin–Elmer AAS Model 103 fitted with an Intensitron hollow cathode Si lamp and nitrous oxide burner was used in all analyses. The operating parameters were: wavelength 251.6 nm, slit width 0.2 nm, lamp current 15 mA, acetylene flow rate 2.4 l/min, nitrous oxide flow rate 4.2 l/min. Blood was allowed to clot in polystyrene tubes and the serum was separated into polystyrene bijou bottles. Syringes and plastic pastettes were found to be free from Si or silicone contamination. Twenty-four hour urine samples were collected in clean 2.5 litre polyethylene bottles.

Sample treatment. Twelve urine samples and 10 pooled serum samples were analysed by direct aspiration (no pretreatment) and by the standard addition method. In the urine analyses the mean Si concentrations for the two procedures were 674 and 686 μmol/l and for the serum 30.1 and 29.9 μmol/l respectively. The pairs of values obtained for each serum and urine sample were not significantly different when subjected to a paired *t* test.

Precision. Day-to-day reproducibility of flame AAS was observed by analysing one urine sample on 26 separate days. The between-batch coefficient of variation (CV) was 4%. For pooled serum sampled and analysed on 20 separate days the between-batch CV was 13%.

Viscosity. We studied serum viscosity by analysing 24 pooled serum samples by direct aspiration using either (1) aqueous Si standards and a blank prepared with deionized water or (2) Si standards and a blank prepared with 10% glycerol deionized water (v/v). The glycerol solution matched the serum viscosity. The aspiration rate of serum and 10% glycerol was 7.8 ml/min. The mean serum Si concentration was 31 μmol/l as measured by (1) and 27 μmol/l as measured by (2). There was no significant difference between serum Si concentrations obtained by these two methods.

Method comparison. Urine samples from 34 healthy adults, including some given 5 g magnesium trisilicate orally, were analysed for Si by flame AAS (x) and by the colorimetric method of Jankowiak & LeVier (1971) (y). Silicon concentrations ranged from 93 to 1816 μmol/l. The regression equation was expressed by $y = 1.01 \, x + 9.48$.

Recovery of Si added to serum. Varying amounts of Si were added to four different pooled sera. Recovery of Si ranged from 80–103% with a mean value of 99% from the 12 samples tested.

Other analytical techniques

Flameless graphite furnace atomic absorption spectroscopy (GF–AAS) provides the high sensitivity and low detection limit required for the microdetermination of Si in biological material. For analysis of serum, Lo & Christian (1978) state that careful temperature control during the ashing phase is of critical importance in preventing loss of Si by volatilization.

Inductively coupled plasma atomic emission spectroscopy (ICP—AES) is reported to be particularly suited to the determination of refractory elements such as Si and is relatively free from chemical interference (Perkin–Elmer 1981).

   Si concentrations in serum and urine as determined by these methods are listed in Table 1.

Absorption and urinary excretion of Si in normal and uraemic individuals

Urinary excretion of Si in groups on different diets

The 24-hour urinary excretion of Si was determined in five groups of individuals on different diets (Table 2, Figs. 1 and 2). There was a highly significant difference between these five groups, with group A showing the lowest excretion among healthy individuals. The highest excretion was found in the patient receiving continuous silicate antacid therapy. The two healthy individuals on a normal diet (group B/C) who were studied over a two-month period showed minor daily fluctuations in excretion of Si, while those in group D, receiving 5 g magnesium trisilicate, showed a 3–38-fold increase above baseline levels in the first 24 hours after silicate ingestion, with Si excretion remaining high for a further two days.

Si absorption and excretion test

Simultaneous measurements of Si concentration in urine and serum were also made at frequent intervals over 24 hours in two healthy subjects, after ingestion of 5 g magnesium trisilicate. Peak urinary concentration (up to 10.68 mmol/l) and peak serum levels (two–threefold above baseline) were found after 1–2 h (Fig. 3).

Serum concentration of Si in random blood samples (normal and uraemic individuals)

The serum concentration of Si was measured in (a) 173 healthy subjects (95 men, 78 women) on a normal diet, not receiving any medicines, and (b) 21

TABLE 1 Silicon concentration in serum and urine using colorimetry and three atomic spectroscopy techniques

| Technique | Mean silicon concentration in health | | Sensitivity µmol/l | Detection limit µmol/l | Comment |
	Serum µmol/l	Urine µmol/l			
Colorimetry	49.8 (King et al)	304 n = 12 (Dobble & Smith)	—	—	Contamination from reagents; laborious method
Flame AAS P–E 103	21.5 ± 4.5 n = 50 (Dobbie & Smith)	194 ± 94 n = 50 (Dobbie & Smith)	43	7[a]	Good precision
GF–AAS P–E 403/2000	27.4 n = 5 (Lo & Christian)	455[b] n = 6 (Lo & Christian)		0.004	Low detection limit
ICP–AES P–E ICP/5000	—	377 ± 171 n = 35 (Minoia et al)	3.56	—	Few chemical interferences; suited to refractory elements

P–E, Perkin-Elmer.
AAS, atomic absorption spectroscopy.
GF, graphite furnace.
ICP–AES, inductively coupled plasma atomic emission spectroscopy.
[a] Current instruments. Detection limit for silicon, 2 µmol/l.
[b] Silicon concentration in drinking water. 178 µmol/l. All samples from 1 subject.
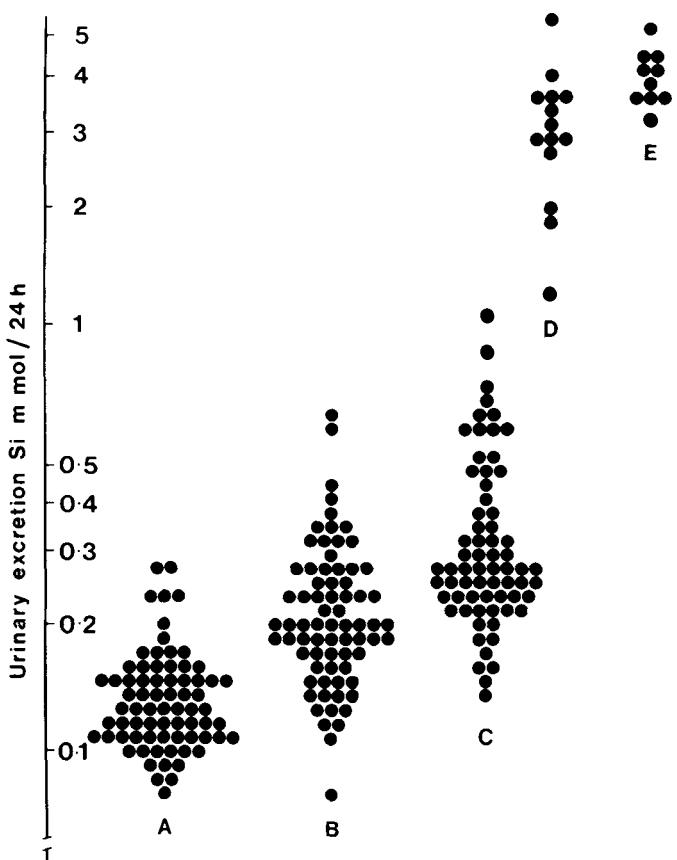
FIG. 1. Urinary excretion of Si (mmol/24 h, semi-log scale) in five groups of adults with increasing dietary intake of Si. A, men on low Si diet (96 μmol Si/day). B (women) and C (men) on normal diet (>360 μmol Si/day. D, subjects given 5 g magnesium trisilicate. E, patient on continuous silicate antacid therapy.

patients (11 men, 10 women) with chronic renal failure (serum creatinine > 450 μmol/l) on a 40 g protein diet, but not receiving any Si-containing medicines.

In random blood samples from the males, the mean serum Si was 21.3 μmol/l), SD±5, range 14–39, and for females the mean was 21.7 μmol/l, SD±5, range 14–39. There was thus no significant difference in serum Si between males and females on a normal diet. In the 21 patients with chronic renal failure, the mean serum Si was 46.3 μmol/l, SD±12, range 36–78. This was significantly higher than that found in healthy adults ($P < 0.0001$) (Fig. 4).
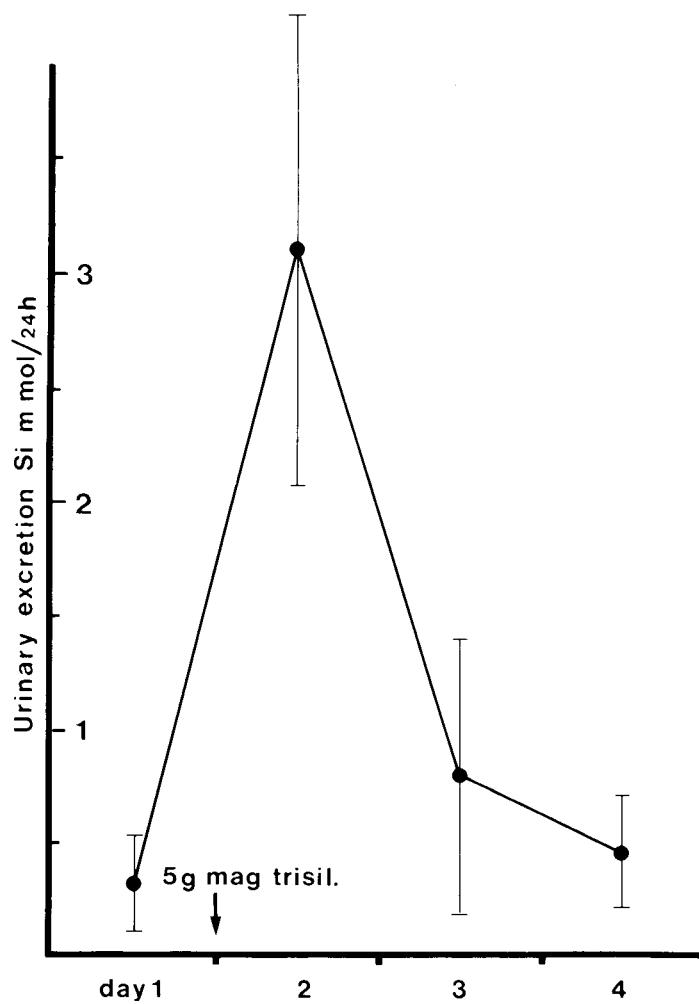
FIG. 2. Urinary excretion of Si (mmol Si/24 h) in 14 adults after a 5 g dose of magnesium trisilicate.

TABLE 2 Urinary excretion of Si in healthy individuals and one patient

Group	No. in group	Sex	No. of 24-h collections of urine	Daily diet	Si in diet μmol/day	Si excreted mmol/24 h mean ± SD (range)
A	9	Male	71[a]	3.4 l milk	96	0.13 ± 0.13 (0.08–0.25)
AA	1	Female	2	Water only	16	0.016
B	72	Female	72	Normal	>360	0.2 ± 0.08 (0.08–0.57)
C	66	Male	66	Normal	>360	0.31 ± 0.15 (0.14–1.13)
B/C	2	1 male, 1 female	12	Normal	>360	0.3 ± 0.06 male 0.27 ± 0.06 female
D	14	5 males, 9 females	14[b]	Normal + 1.6 g Si[c]	>360	3.1 ± 1.02 (1.1–5.34)
E	1	Male	10	Normal + Mg trisilicate[d]	>360	4.0 ± 0.57 (3.1–5.0)

[a] Collected over two periods of four consecutive days.
[b] Collected on four consecutive days; pooled for each individual.
[c] A single dose of magnesium trisilicate BP, 5 g, containing not more than 1.6 g elemental Si, was taken for the beginning of the second 24-h collection period.
[d] This patient was taking magnesium trisilicate continuously as antacid therapy.
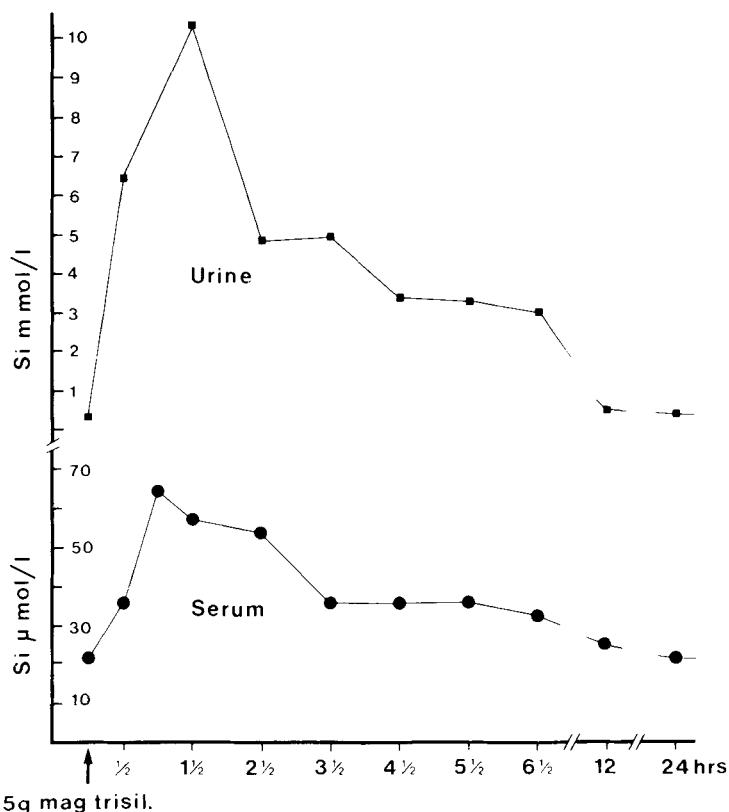
FIG. 3. Simultaneous measurements of Si concentration in urine (mmol/l) and serum (μmol/l) in a single individual after ingestion of 5 g magnesium trisilicate.

Urinary excretion of Si in renal disease

In 184 patients with renal disease and varying degrees of renal impairment, the 24-hour urinary excretion of Si and urinary concentration of Si decreased with deteriorating creatinine clearance (Table 3).

Serum Si in patients on regular haemodialysis

Serum Si was studied in four groups of patients

Group 1: Glasgow Royal Infirmary, low tap water Si (18 patients).
Group 2: Western General Hospital, Edinburgh, intermediate tap water Si (5 patients).

TABLE 3 Daily urinary excretion of Si in 184 patients (78 men, 106 women) with renal disease

No. of patients	GFR	Mean urinary Si excretion mmol/24 h ± SD	Range (mmol/24 h)	Mean urinary Si concentration μmol/l±SD	Range μmol/l
57	<20	0.136 ± 0.057	0.028 ± 0.267	83 ± 27	36–164
45	20–60	0.245 ± 0.078	0.110 ± 0.523	137 ± 60	57–267
40	60–100	0.216 ± 0.063	0.075 ± 0.393	143 ± 59	46–320
42	>100	0.288 ± 0.114	0.096 ± 0.822	211 ± 104	68–605

GFR, glomerular filtration rate.
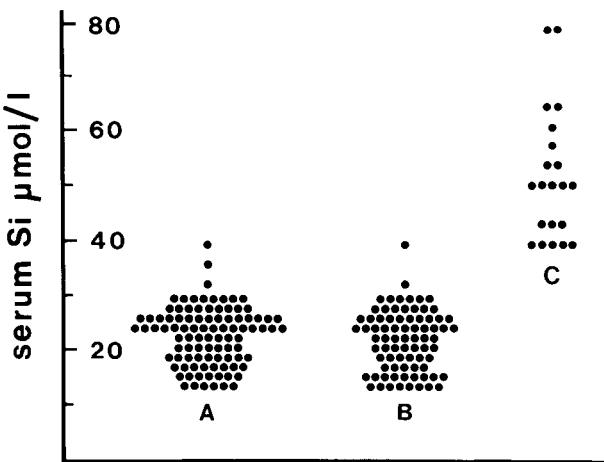
FIG. 4. Serum concentration Si (μmol/l) in random blood samples from 95 healthy men (A), 78 healthy women (B), and 21 patients (11 men, 10 women) with chronic renal failure (C).

Group 3: Edinburgh Royal Infirmary, intermediate tap water Si. Reverse osmosis used (19 patients).

Group 4: The London Hospital, Whitechapel, London: High tap water Si (16 patients).

Si concentration was determined in the water supply, dialysate and immediate pre-dialysis and post-dialysis serum (arterial sample). In Group 3, the Si concentration of dialysate before and after reverse osmosis was determined. In 47 of the 58 patients, the duration of dialysis was five hours, in six patients it was six hours, in four patients four hours, and in one patient three hours. None of the patients was receiving Si-containing medicines, and the Si content of heparin was found to be insignificant.

The concentration of Si in tap water, dialysate, pre-dialysis and post-dialysis serum for each group, together with the Si concentration of the dialysate before and after reverse osmosis in Group 3 are listed in Table 4, while Fig. 5 shows the changes in serum Si during haemodialysis for each patient.

In Glasgow, where the dialysate Si concentration (20 μmol/l) was lower than the mean serum Si concentration of normal subjects (21.5 μmol/l), serum Si fell during dialysis and rose between dialyses to levels found in non-dialysed patients with chronic renal failure. In Edinburgh, a region of intermediate water Si concentration where reverse osmosis was not used, serum Si equilibrated with a dialysate Si which was four times that in Glasgow. In the Edinburgh Royal Infirmary, it is worthy of comment that reverse osmosis achieved only a 55% reduction in Si concentration in the tap water. Thus, the Si

TABLE 4 Si concentrations in tap water, dialysate and serum in 58 patients on haemodialysis in Glasgow, Edinburgh and London

Location and no. of patients	Tap water Si µmol/l	Dialysate Si µmol/l		Mean serum Si µmol/l ± SD (range)		Mean % change in serum Si
		No R.O.	After R.O.	Pre-dialysis	Post-dialysis	
Glasgow Royal Infirmary (n = 18)	14	20	—	45 ± 7 (28–57)	30 ± 5 (25–43)	−33
Edinburgh Western General Hospital (n = 5)	78	80	—	81 ± 16 (68–107)	85 ± 8 (78–100)	+8
Edinburgh Royal Infirmary (n = 19)	69	75	34	59 ± 12 (39–82)	37 ± 8 (28–57)	−35
London (n = 16)	142	186	—	149 ± 17 (121–174)	173 ± 19 (139–217)	+16

R.O., reverse osmosis: 1 µg Si/ml = 35.6 µmol/l.
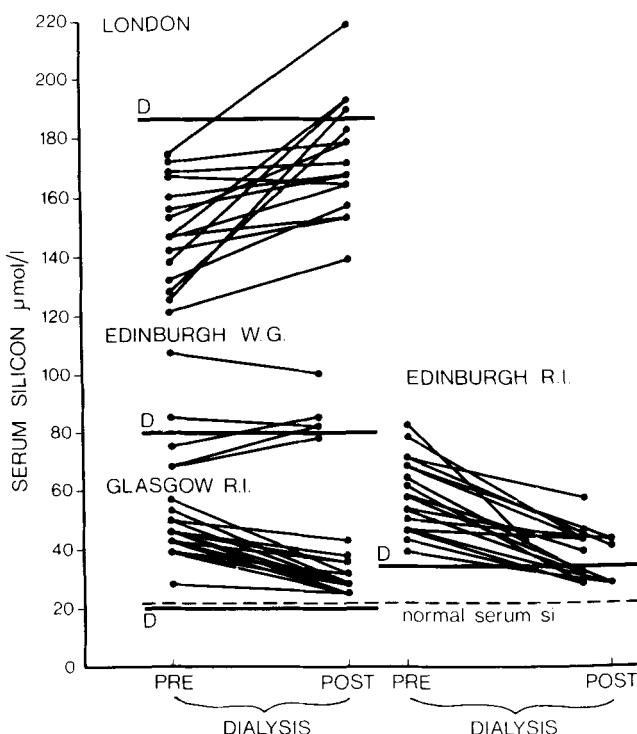
FIG. 5. The changes in serum Si during haemodialysis for each patient in four centres. Continuous lines (D) are the Si concentrations in the dialysate at each centre. The broken line represents the mean serum Si of normal individuals in Glasgow.

content of dialysate remained above that of normal serum Si, and although Si levels fell during dialysis, the post-dialysis serum Si concentration in this group of patients was 30% higher than that of patients treated in the region where dialysate was constituted with naturally lower Si water. In London, where dialysate Si was eight times that of normal serum, serum Si rose during dialysis and fell between dialyses.

Serum Si in patients after renal transplantation

Serum samples were obtained from 34 patients (20 men, 14 women) at least three months after successful renal transplantation in Glasgow (serum creatinine range 93–408 μmol/l, mean 160 μmol/l, SD±65). These patients showed a highly significant fall in serum Si (mean 20.76 μmol/l, SD±6.6) compared to a similar group (Group 1) on regular haemodialysis in Glasgow ($n = 18$, mean 45.75 μmol/l, SD±7.1; $P < 0.001$).

Discussion

These studies indicate that urinary Si is mainly derived from dietary intake and that, on a normal diet, humans excrete a surprisingly large amount of Si in their urine. In health, serum Si is maintained within a relatively narrow range, although a twofold–threefold increase can occur briefly during silicate ingestion.

With increasing renal functional impairment, urinary elimination of silicon decreases and serum silicon rises. The concentration of Si in the serum of uraemic patients (serum creatinine > 450 μmol/l) is consistently two to three times that found in normal healthy individuals. Thus, hypersilicaemia should now be recognized as a further biochemical derangement characteristic of the uraemic state. After successful renal transplantation, the raised levels of serum silicon fall to near-normal values by three months.

The findings in dialysed patients demonstrate a close correlation between Si concentration in the serum and dialysate for each centre studied, suggesting that the Si content of the water supply is the major factor in the geographical variation in serum Si in dialysed patients. Thus, Si is removed in Glasgow, where the water is naturally low in Si and at Edinburgh Royal Infirmary where reverse osmosis is used, but is dialysed into patients at the Western General Hospital and in London where the Si content of tap water is, respectively, intermediate and high.

The systemic effects of uraemic hypersilicaemia are unknown. However, the available information on the biological role of Si suggests that alterations in the metabolism of the matrix of connective tissue, bone and skin might reasonably be expected. Analysis of the patterns of morbidity in haemodialysed patients in the three regions where serum Si is twice, four, and eight times normal may therefore contribute to our understanding of the biological effects of Si in humans.

Uraemic hypersilicaemia may also allow Si to protect haemodialysed patients from the pathogenic effects of aluminium dementia. A rough epidemiological survey we have made of incomplete data gathered from a variety of published and unpublished sources in Western Europe suggests that there may be a higher incidence of dialysis dementia in areas with soft water (low dialysate Si) and a lower incidence in areas with hard water (high dialysate Si). It is possible that dementia does not occur unless serum aluminium levels exceed serum Si concentrations. It would seem that an accurate epidemiological survey of the geographical incidence of dialysis dementia before and after the introduction of reverse osmosis is called for.

This new information on the renal handling of Si may stimulate interest in previous suggestions that Si is involved in the causation of several renal diseases. The high peak concentrations of Si found in the urine during silicate loading may be of significance in the formation of renal stones. Silica urolithiasis occurs in cattle fed for long periods on silica-rich fodder (Keeler 1963),

while it has been recognized that patients on silicate antacids can produce pure silica stones (Joekes et al 1973). Silicic acid is known to inhibit the action of the peptide inhibitors responsible for the prevention of mineral crystallization in urine (Thomas 1969). The possible role of urinary Si in the initiation of all forms of renal stones has been largely overlooked, although it has been suggested that the high concentration of Si in hard water may be as important as its calcium content (Thomas 1969).

Suspicion of Si as a potential nephrotoxic agent in humans comes from its suggested role as a causative factor in Balkan nephropathy (Marcovic & Arambasic 1971), from earlier work on the incidence of renal lesions in silicosis (Kolev et al 1970), and from several reports of renal damage after industrial exposure to Si dust (Saldanha et al 1975, Hauglustaine et al 1980, Giles et al 1978). Silicates, a frequent constituent of proprietary analgesics, have been ignored as a causal agent in analgesic nephropathy despite evidence that their long-term administration produces tubulo-interstitial lesions in the kidney in experimental animals (Dobbie & Smith 1982b).

Si occurs naturally in many foodstuffs and beverages, particularly vegetables, cereals and beer. The Si content of drinking water shows geographical variation, being high in hard water and low in soft water areas. In recent years, silicate additives have been increasingly used in prepared foods and confections as anti-caking agents. In the United Kingdom, the regulations governing silicate additives permit the use of a large range of compounds, up to a maximum of 2% by weight (Ministry of Agriculture, Fisheries & Food Report 1968). The committee on medical aspects of food policy has stated that the toxicological evidence on silicates was not entirely satisfactory in view of the absence of long-term studies.

After years of indifference, the biological role of Si in humans is now receiving increasing attention. These studies emphasize the crucial role of the kidney in the understanding of the metabolism of Si in health and disease.

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DISCUSSION

Hench: Have you any comments on the possible role of silicon in urolithiasis?

Dobbie: High levels of silicon can be produced in human urine. Silicon stones have been found in the urinary tract of American beef cattle and in sheep. There are several case reports of silicon stones in humans. We measured silicon excretion in 50 stone-formers compared with 50 normal individuals who were carefully matched. We found a statistically significant increase in silicon excretion in the stone-formers (Dobbie & Smith 1982). Thomas (1969) suggested that silicon may inhibit the peptide inhibitors of stone formation, which seems a reasonable supposition. From what we have seen of the urinary excretion of silicon I am not surprised at the explanation given in the earlier reports on silicon stones in cattle.

Volcani: Are silicate-binding proteins involved in the formation of these stones?

Dobbie: I think Dr Thomas was suggesting that this might be the case.

Hench: The ultrastructure of more typical oxalate stones in humans shows, in addition to the crystalline arrays, a very amorphous matrix phase of only 1–4 nm in diameter. At one time it was thought that that layer might have silicon in it. Was it ever proved whether silicon is involved in the formation of stones in stone-formers?

Carlisle: In animals it is thought that a glycoprotein and magnesium are involved in initiating urolithiasis. Until a few years ago silicon was reported to occur in stones in only about nine or ten people in the world. However, many laboratories do not analyse for silicon, only being concerned about calcium, phosphate and magnesium.

Hench: But has the amorphous binding substance in the ultrastructure been identified?

Carlisle: I can't answer that.

Dobbie: I think Thomas (1969) said that in the stones that he had analysed there was a fixed amount of silicon for each stone analysed.

Hench: He has not looked at the ultrastructure, as far as I know, but Finlayson has (Finlayson et al 1972).

Last: Is the human urine concentrating silicon against a gradient or is it reaching equilibrium with the blood level?

Dobbie: It must be concentrating against the gradient because the levels in the distal tubules are very high compared with the twofold to threefold increase seen in silicon loading. I don't know what the tubular physiology of it is. The methods using a graphite furnace are very sensitive. I don't know whether those could be used in microtubule studies.

Farmer: You showed that ground quartz caused renal failure when it was fed to guinea-pigs. If you grind quartz you create amorphous areas on the surface and if you then put it into water the level of silicon in solution goes up to the same level as if it was amorphous silica. To get back to the true solubility of quartz you have to clean up the surface with hydrogen fluoride. I don't think your evidence supports a direct effect of quartz on the kidney.

Dobbie: That is exactly what we felt. None of this quartz got into the kidney. We measured the supernatant and assumed that this was all soluble material which was absorbed into the blood stream. Marcovic & Arambasic (1971) thought likewise. The levels we find in these experiments are never approached in the normal environment.

Perry: How much silicon did you detect in the membranes?

Dobbie: The peak was found over the cell membrane of the luminal surface of the distal renal tubule. There was also a small amount in the basement membrane in the kidney.

Perry: Is it really what we term soluble silicon or is it colloidal material attached to the membrane?

Dobbie: It is there because it is mobile but once at this site it becomes

attached to cell membranes. Could any rigidity develop which constrains the membrane?

Mann: In vesicle experiments we had very high supersaturations. However, in the 1950s Clark and co-workers set out a Langmuir monolayer membrane system with silicic acid underneath the monolayer (Clark et al 1957). As they brought the barrier in they increased the surface pressure and consequently decreased the headgroup spacing of the monolayer. The results suggested silica polymerization at the interface. The induction of silica deposition seems to indicate specific binding at the membrane surface, such that at a particular spacing polymerization at the interface is initiated.

Dobbie: That fits in with the contention that alterations in cell permeability occur when soluble silica attaches to cell membranes.

Birchall: Körösy & Taboch (1973) looked at the electrical properties of lecithin membranes in acidified sodium silicate. The electrical properties changed only after a certain time, indicating that the silica had to polymerize before it altered the membrane structure. It is not the monomer but the polymer that damages membranes.

Perry: Even in unfavourable conditions for silica deposition, i.e. pH 2, deposition occurs very readily in the presence of phospholipid vesicles.

Dobbie: So you think this may be happening in affecting permeability of the cell membrane?

Perry: It is a possibility, because under normal fixation conditions all soluble material is lost.

Dobbie: I am cautious about what is inside the cell. The techniques are now available to demonstrate silica in unfixed, snap-frozen samples.

Werner: You mentioned that perhaps silica affects the membranes. Could a peroxidation of membrane lipids be involved?

Dobbie: You have just seen the evidence for that. These cells show damage associated with membrane permeability problems. They are poisoned with silica. There is certainly an association between changes in the tubular permeability associated with high local concentrations of silicon in the lumen.

Werner: Permeability is of course a process through membranes but the conclusion that the target is the membrane itself is not necessarily the only possibility.

Dobbie: The silicon was stuck on the outside. We did this expecting to see silicon in organelles in various patterns but only found cells damaged by oedema.

Richards: They are epithelial cells so you wouldn't expect silicon as a particle to go in anyway.

Dobbie: These cells indulge in micropinocytosis. Their job is to put material in and out.

Richards: Presumably the particles may not be coated in the right way to get completely in.

Espie: A recent paper on the interactions of protein monolayers spread on silicic acid substrates (Minones et al 1984) confirms that there are specific interactions between positively charged protein groups and the negatively charged ionized silanol groups giving mainly ionic rather than hydrogen bond interactions which could be important in altering membranes.

Dobbie: This is getting back to what is happening when the crystalline material is in the lysosome, where it could be stuck to the membrane which is held rigid.

Edwardson: Is it your final opinion that environmental exposure is a contributory factor in Balkan nephropathy?

Dobbie: The jury is still out. It is not necessarily caused by silicon.

Edwardson: What about the silicon levels in the water?

Dobbie: In some of the areas it is high, in others it is not.

Edwardson: Dr Farmer gave us a picture of relatively steady levels of silica in river water, hardly varying, with quite a small range. Are you saying there are levels which exceed those he gave?

Dobbie: The silicon concentration is just one of many explanations of this fascinating condition. The others vary from toxins from *Aristolochia clematitis* to coronaviruses from pigs. Silicon is quite low on the list.

Edwardson: Were the silicon levels above average?

Dobbie: Yes.

Farmer: The data you quote for London tapwater are right in the middle of the range for river water world-wide. The low figures for Glasgow could be either because the water has been treated with aluminium or because it is draining off peaty surface soils and is not passing through the mineral soil at all.

Dobbie: Some of the water was collected from a pure granite area and had very low levels of silicon.

In aluminium dementia, the ratio of aluminium to silicon in the serum varies but may be 1:1 or 1:2. Serum aluminium is usually measured every month in dialysis units in the UK. At 2–3 μmol/l there is no problem but at 6–7 μmol/l there is concern. In the early days some of the levels went up to 12–13 or 17–18 μmol/l. In one patient we found about 30 μmol/l. The silicon level in that patient was 35–40 μmol/l.

Edwardson: But you can't measure the brain content under those conditions.

Dobbie: The battle may have been lost before aluminium or silicon gets to the brain. Concentrations like this are not normally found in any living mammal, putting aside the Alzheimer figures. These concentrations are unique. I am trying to make some sense of the aluminium:silicon ratio in the dialysis patients.

Edwardson: The brain is unique in that it has a blood–brain barrier to protect itself from aluminium and silicon. The plasma levels can be very high but unless you can say what the levels are in the extracellular fluid in the brain I don't see that the ratios have much physiological significance.

Dobbie: I have measured silicon in the CSF. The blood–brain barrier is certainly not intact at all times.

Edwardson: It is probably not intact in Alzheimer's disease but are we dealing with a small overload over several decades or with episodic exposure to much greater levels within a shorter time scale?

Dobbie: I don't know. Vascularity degenerates with age. Hypertension increases the rate of damage. Microaneurysms show up in hypertensive patients as they age. As the person ages, the blood–brain barrier also ages. If there is an abnormal external environment in the way of tissue fluid and blood, anything can happen.

Edwardson: The incidence of coexisting Alzheimer's disease in patients with multi-infarct dementia is greater than one would expect. This is consistent with a hypothesis involving exposure to a toxic agent after breakdown of the blood–brain barrier.

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Silica and oesophageal cancer

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Abstract. The growth of animal cells in culture can be stimulated very powerfully when they are allowed to extend upon a solid surface. In normal fibroblasts, the maximum is reached either on a plane surface with an area of 2500 μm^2 or on a narrow fibre with a length of 250 μm. This growth-stimulating effect of fibres could help to explain how asbestos causes cancer. All asbestiform minerals are complex mixtures of different lengths, but siliceous macrohairs with a uniform length are borne by several species of the grass genus *Phalaris*. Some of these species are common contaminants of the bread eaten in a part of Iran where oesophageal cancer has an unexplained high incidence. A pure preparation of 200 μm silica fibres from one of these species is a powerful promoter of cancer in the skin of mice. Similar fibres from millet (*Seteria italica*) are associated with the same disease in China, and plant silica has long been known to be associated with it in South Africa. In addition, a rare thoracic tumour, which normally only occurs after exposure to asbestos, has been detected among sugarcane farmers in the United States and in India; fine silica fibres are liberated into the air during the harvest.

1986 Silicon biochemistry. Wiley, Chichester (Ciba Foundation Symposium 121) p 214–230

Evidence that biogenic silica is involved in human cancer is beginning to accumulate. Various kinds of contamination with fibrous silica have been discovered in the diets of three populations in Asia and Africa who experience extremely high incidences of oesophageal cancer, and one of these contaminants has been purified and found to promote cancer in mice. Biogenic silica is therefore a new cause of cancer. It is still far from usual to look for it in the human diet or environment, and we have no idea yet how important it might eventually turn out to be.

We give here a brief description of our work on the proliferation of animal cells and how it has led us to suspect that fibres of a particular size (either mineral or biogenic) might act to cause cancer. We show how fibres of biogenic silica of this size contaminate the diets of populations at high risk for oesophageal cancer, describe the isolation of one of these fibres in essentially pure form, and show how it can produce cancer in mouse skin under standard

experimental conditions. Finally, we list those other biogenic silica contaminants of the environment which can be associated with unusual incidences of cancer in humans or in domestic animals, and describe the technical problems which face us.

Causes of cancer

In general, we do not know what causes cancer. Most human cancers are still unexplained, and tobacco contributes significantly only to the incidences seen in Europe or North America. Environmental contaminants produced by industry, such as asbestos, cannot account for more than 10% of the cancers seen even in those countries where they are common (Doll & Peto 1981). Some of the highest incidences are now known to occur in rural parts of Africa and Asia (Waterhouse et al 1975). However, it seems clear that the causes are environmental, in the sense that the incidences of different cancers differ by factors of 10 or 100 between different countries. Migrant populations eventually become free of the tumours characteristic of their parent countries (Cairns 1979, Cairns et al 1980). It therefore seems that it is preventable; our problem is to identify those elements of the environment, diet or lifestyle which give rise to it.

Cancer is not (except in rare instances) an inherited disease, but it is a genetic one. The controls of growth and differentiation which regulate the cells of healthy animals are complex and robust; a long process of somatic selection and evolution is necessary before one clone of cells eventually becomes autonomous and grows to destroy its host (Klein & Klein 1985). Cancer is caused by agents which can make this somatic selection happen. These agents are of two kinds, either mutagens or mitogens. That is, they are either agents which cause changes in the genome, or agents which stimulate proliferation so that selection among these changes can take place. In a typical experiment, an 'initiator' (almost all initiators can be shown to have mutagenic properties) is first applied to the skin of a mouse. A 'promoter' (which will generally have obvious mitogenic properties) is then painted on repeatedly. Well-known examples of initiators are polycyclic hydrocarbons such as benzo[a]pyrene which attack the DNA sequence chemically. The most powerful promoters known are the phorbol esters, a group of plant diterpenes which mimic the growth-promoting effects of the endogenous diacylglycerols on protein kinase C, one of the regulatory mechanisms referred to above. We still have no idea of the relative importance of these two types of aetiological agent to human cancer in real life.

Surface area can regulate cell growth

One approach to the study of the regulatory mechanisms controlling growth is

to explant human or animal cells from tissues and grow them in culture dishes. Even under these simple conditions the cells show a very strict regulation. When they are seeded into a dish they rapidly attach, spread, start moving and grow out to colonize the surface, but all this growth ceases when a confluent sheet is formed. If the cells are then detached and seeded into a fresh dish, the same process occurs again. Nutrients and hormonal stimulators of growth are necessary, but not sufficient; even the medium from quiescent confluent cultures still retains the ability to support growth if it is applied to freshly seeded cells. The regulatory mechanism seems to depend on the amount of solid substratum available, since cells which are prevented from attaching to the surface of the dish will cease to grow almost immediately. In general, suspended cells can only be induced to grow if they have been derived from
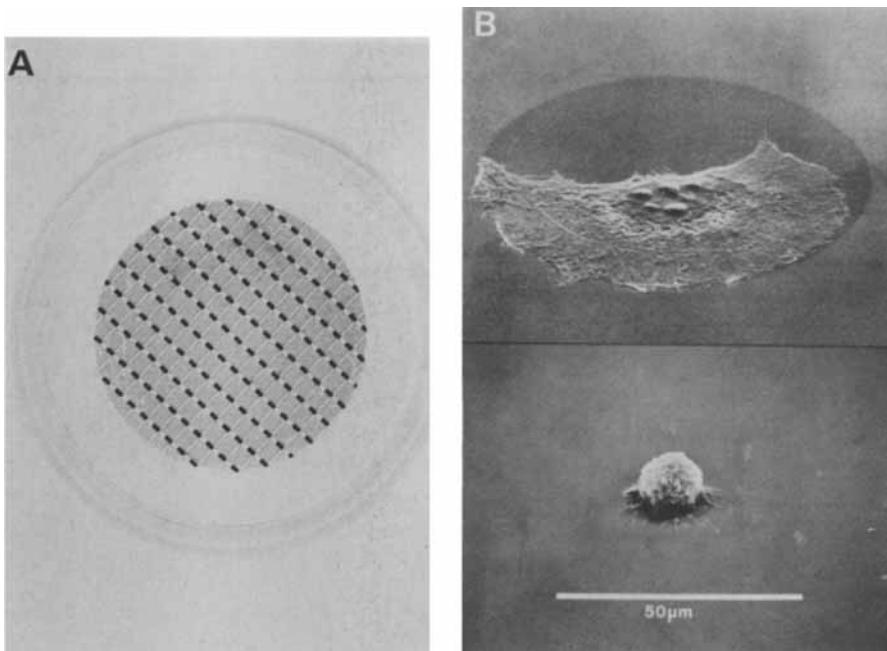
FIG. 1. (A) Dish bearing an array of adhesive islands, as described in the text. The islands range between 400 and 5000 μm² in area. In addition, larger areas (0.75 μm long) of unobstructed substratum are present; it is only these larger areas which can be seen clearly at this magnification. The islands are vacuum-evaporated in palladium onto a non-adhesive substratum of HEMA (polyhydroxyethylmethacrylate).
(B) Cells of the 3T3 mouse line attached to islands of 5000 μm² (upper picture) and 400 μm² (lower picture). Separation between the island centres is 150 μm, and hence only one island can be pictured in each case. The cells are from an active culture in random growth.

tumours, or their genomes have been altered by carcinogens or by the introduction of tumour cell DNA. Growth in suspension is a useful way of assessing the tumorigenicity of experimental cells and is often called anchorage independence.

We have devised a way of measuring this dependence on the substrate by offering individual cells a graded series of minute islands of adhesive substratum. These islands are surrounded by a surface which does not allow the cells to attach (made from the same material as soft contact lenses) (Folkman & Moscona 1978). In this way, single cells can be kept in isolation and yet offered the whole range of substrate areas which they would encounter during their growth towards confluence (Fig. 1A). At its most extreme, the size of the island can be so small that the cells are forced to adopt a hemispherical shape (Fig. 1B). Only the size of the substratum can change; the possible effects of cell
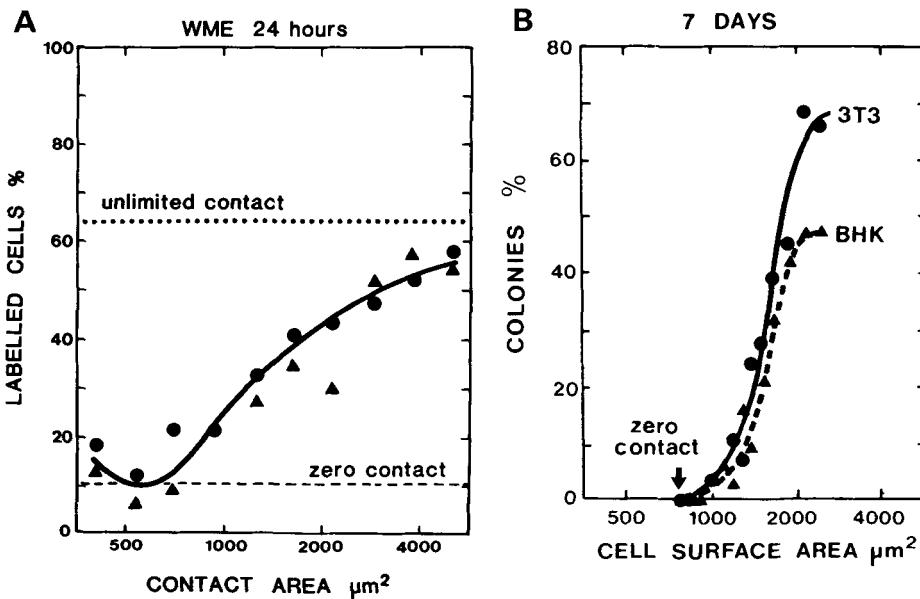
FIG. 2. (A) Proportion of whole mouse embryo (WME) fibroblasts undergoing DNA synthesis as a function of the size of island to which they are attached. Incubation is for 24 hours in growth medium containing [^{3}H]thymidine, and the percentage of cells incorporating thymidine is recorded for each island size. Different symbols refer to different experiments. The horizontal interrupted lines refer to cells allowed unlimited attachment on unobstructed substratum (. . . .), and cells denied any attachment at all, and maintained in suspension in soft gel above the island array (– – – –).
(B) Proportion of 3T3 and BHK fibroblasts stimulated to form colonies by fine glass fibrils. The surface area initially exposed by single cells is calculated from the fibre length on the assumption that the cells adopt an ellipsoidal shape whose volume is 2000 μm^3. 'Zero contact' indicates the surface area of unattached cells.

movement, encounters with other cells and general depletion of the medium can be discounted. These islands are imprinted over the whole surface of a culture dish so that the number of cells is the same as in an ordinary freshly seeded dish. Because the islands are so numerous, our measurements can be quite precise.

The results of a typical experiment are shown in Fig. 2A. This plots the proportion of cells which undergo DNA synthesis in preparation for division, as a function of island size. In addition, we include the proportions of cells kept in suspension above the surface of the dish which also undergo DNA synthesis, and similarly the proportion of cells which undergo DNA synthesis when they are allowed unlimited attachment. The experiment shows that the proportion of cells synthesizing DNA on some of the smallest islands can be the same as that seen in suspended cells. In each case, it is about 10% of the total. It is also interesting to note that the curve rises significantly at its lower end; this is a consistent feature of many different experiments.

Both these features are what would be predicted if the surface area of the cell exposed to the medium were the controlling factor. Knowing the volume of the cells, we can calculate their surface area when they adopt a spherical shape in suspension, and similarly the area of the upper surface exposed to the medium on the various sizes of island. These two figures reach equality on islands of about 700 μm^2. Ordinary considerations of geometry show that when cells adopt a hemispherical shape they expose about 20% less surface area than spheres of equal volume. Further reduction in island size should cause the exposed surface area to begin to increase slightly in just the way that is observed. Hence, both the effect of progressively decreasing island size and the relative numbers of suspended and attached cells which undergo DNA synthesis can be predicted from the surface area of the cells. This experiment therefore shows that it is the surface area of the cell which determines its rate of growth in culture.

This brief description cannot, of course, be a complete account of the complex field of studies of growth regulation. It must also be remembered that only a few of the various kinds of tissue cell can yet be cultivated outside the body. Nevertheless, the general principle that an increase in surface area can give a powerful stimulus to growth seems quite secure.

One consequence of this principle is readily open to test. If surface area is the critical parameter, then spreading in one dimension should be as effective as in two. If cells extended along a line or a rod, their surface area could be equally increased, and they should be equally stimulated. This can be tested by allowing the cells to attach to glass fibres less than 1.0 μm thick, suspended in soft gel culture medium (Maroudas 1973). In this case it is more difficult to detect DNA synthesis, but it is simple to determine the proportion of the fibres which subsequently give rise to large colonies of cells. Two experiments of this sort are shown in Fig. 2B, which shows the response of 3T3 mouse and BHK

hamster fibroblast cells to different lengths of fibre. In this figure, we have plotted colonies against the initial surface areas of the cells when they first attach. The area of the fibre in contact with the cells is minute in comparison with the islands we used before. It can be seen that the proportion of the cells stimulated to grow by fibres in each case reaches a maximum when the surface area is about 2500 μm^2. This area is little different from the area exposed by cells on the islands described above when maximum growth was stimulated. It is strong confirmation of the idea that surface area determines growth in these cells (O'Neill et al 1986).

Asbestos and cancer

One immediate consequence of this idea is that it might explain the way in which asbestos causes cancer. Perhaps it stimulates the proliferation of whatever cells it touches, by allowing them to spread and so increase their surface
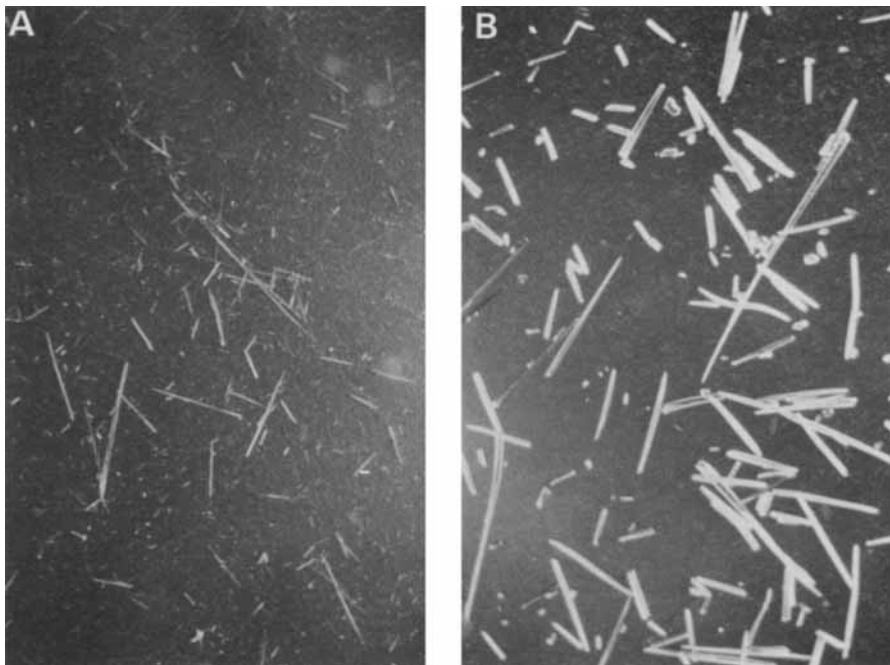
FIG. 3. Comparison of the appearance of carcinogenic mineral and biogenic silica fibres. (A) Crocidolite, standard sample from the Union International Contre le Cancer. (B) *Phalaris canariensis* from Queensland, isolated as described by Bhatt et al (1984). The fibres are suspended in water and visualized by differential interference contrast. Both pictures represent fields measuring 1.0 mm × 1.5 mm.

area. In this view, asbestos fibres act as promoters rather than initiators, and it is their mitogenic properties which allow them to cause cancer. One way of testing this hypothesis is to determine the lengths of fibre which are effective. The fibres used in the experiment described above first began to have an effect at 50 μm, and they reached their maximum stimulating power at 250 μm. It should be these lengths which cause cancer, if we suppose (not unreasonably) that the cells which they attack have similar volumes to the cells we have studied here. Unfortunately, all the fibrous carcinogenic minerals, both native and manufactured, are extremely heterogeneous in size. A standard preparation of crocidolite is shown as part of Fig. 3A. A substantial proportion of fibres
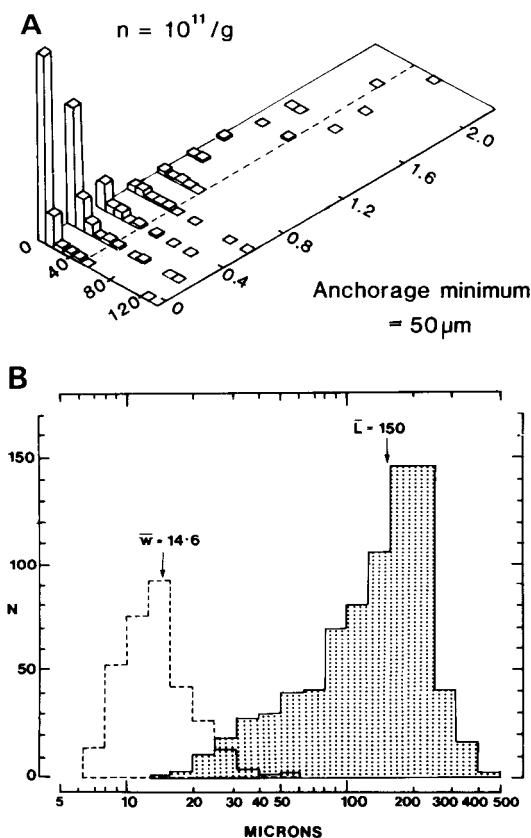
FIG. 4. Size distributions of carcinogenic mineral and biogenic silica fibres. (A) Tremolite, adapted from Wagner et al (1982). The dotted line indicates a length of 50 μm, the minimum found to cause anchorage stimulation of growth as described in the text. (B) *Phalaris canariensis* from Queensland; in this case diameter is independent of length and no attempt has been made to associate them. The hatched area shows length, and the clear area shows diameter. Mean length = 150 μm; mean width = 14.6 μm.

are clearly over 250 μm in length, but a typical size distribution of a carcinogenic mineral fibre (in this case tremolite) shows that most fibres are less than 10 μm (Fig. 4A) (Wagner et al 1982). There is clear evidence that the material of fibrous carcinogens is not important so long as they are durable and sharp, but the idea that they might be most effective at lengths greater than 250 μm is quite novel. It occurred to us that contaminations of the environment with fibres of this size might hitherto have gone unnoticed.

Oesophageal cancer

Oesophageal cancer reaches extremely high, unexplained, incidences in several parts of Africa and Asia, including the Turkoman region of North-East Iran (Cook-Mozaffari 1979). A cursory examination of the grain which is the staple diet here showed us that it was contaminated with the seeds of several species of the grass genus *Phalaris* (O'Neill et al 1980). All these seeds bear pointed siliceous macrohairs about 15 μm in diameter and over 250 μm in length. The tip radius is about 0.25 μm. Fortunately, one of these seeds ('canary grass'; *Phalaris canariensis*) is commercially available and is used as a fodder crop in arid areas. The grain merchants take care to remove as many of the macrohairs as possible because the hairs are extremely irritant to human skin (Wrigley et al 1980). It is simple to make pure preparations of these hairs from the contents of dust-extraction bags. When we treated these hairs in hot nitric acid, the product was a very pure preparation of biogenic silica fibre (Mann et al 1983).

The appearance of our preparation is shown in Fig. 3B, and its size distribution in Fig. 4B. It is a fine pure white flocculent powder which can be rubbed on the skin of mice with no obvious effect. The mice live out their lifespans in apparently robust health. However, if the groups of 20 mice are first treated with a subminimal dose of an initiating carcinogen, most of them develop tumours, as shown in Fig. 5 (Bhatt et al 1984). These silica fibres are therefore an unusual form of tumour promoter. In quantitative terms, they are fully as effective as phorbol esters, and they promote the appearance of more than twice as many tumours; although the tumours themselves are rather slower in appearing and smaller in dimension, the proportion which go on to form invasive carcinomas seems to be the same. Both the pure acid-treated fibre (free from any organic material) and the native grain are equally effective; the presence of the carbohydrate cell wall which must have originally enveloped the native fibre seems irrelevant to its tumorigenicity.

This is the first demonstration that fibres can act to cause cancer by promotion. Mineral fibres can only be shown to cause cancer in soft tissues, perhaps because their relatively narrow diameter renders them too fragile to penetrate the tough keratin layer of the skin, and soft tissue tumours are slow to detect and difficult to analyse.

The same disease, oesophageal cancer, reaches similarly high incidences in a
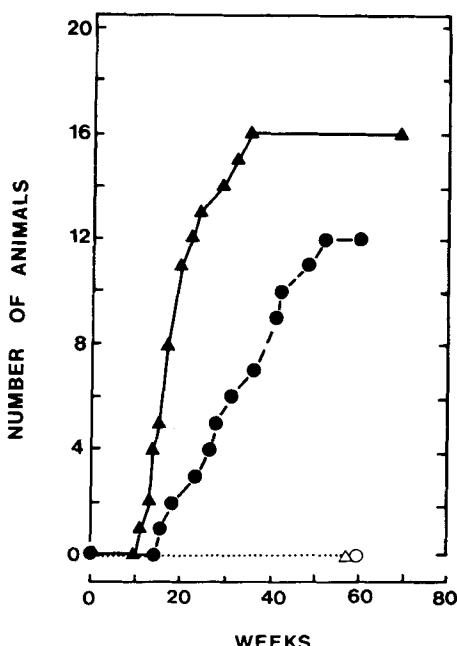
FIG. 5. Incidence of tumours initiated in mice when the skin was painted with 0.4 μg of 11–methyl–15–cyclopenta [a] phenanthrene seven days before the animals were treated with croton oil or *Phalaris canariensis* fibre. Triangular symbols: promotion with 0.1 mg of croton oil (1% in toluene) painted on the skin twice weekly. Circles: promotion with acid-purified fibre, rubbed on twice weekly. Open symbols: no initiator. Only the number of animals bearing tumours is shown (16 with croton oil, 12 with *P. canariensis* fibre); number of tumours per animal is twice as high with fibre as with croton oil.

part of North China. Here it is closely localized in the uplands around the Tai Han Shan mountain range. In this region there is (or was until recently) a traditional dish made from the bran of millet (*Setaria italica*) which consists chiefly of the outer coat of the grain but also includes the macrohairs which cover the inflorescence pedicels. These pedicel hairs are siliceous and seem as fine and sharp as those of *P. canariensis*; in addition, the bracts coating the grain readily shed thin siliceous sheets, and the total weight of silica in the bran is as much as 20% (O'Neill et al 1982, Hodson & Parry 1982, Parry et al 1984). Another localized area of high incidence occurs in the Transkei region of South Africa; in the diet of this region there are several broad-leaved plants, including *Bidens pilosa*, with high silica content (Rose & Guillarmod 1974). The anatomy of these plants has been summarized (Parry et al 1984), and *Bidens* has been found to have a small promoting effect on carcinogenesis in the oesophagus of rats initiated by methyl-*n*-amylnitrosamine when it is included in the diet (Mirvish et al 1985).

Other forms of cancer

A very different and theoretically interesting association between silica and cancer in humans has been reported from India (Miraj) and Louisiana. In both places, a few cases of mesothelioma have been diagnosed among sugarcane farmers (Das et al 1976, Rothschild & Mulvey 1982). This disease is interesting in spite of its general rarity because of its apparent confinement to people who have been exposed to mineral fibres (Cochrane & Webster 1978). It has now been found that sugarcane is heavily silicified, and that fine silica fibrils are liberated in the ash produced by burning the leaves (Newman 1983). The leaves are customarily burnt *in situ* to make harvesting easier, and the cane itself is used as fuel when the sap is boiled to extract the sugar. The fibres are often below 1 μm in diameter, and so are small enough to be breathed into the lungs; a substantial proportion of them are greater than 250 μm in length.

There are several other possible associations which deserve examination. Epidemiologists in the south of Brazil (Rio Grande do Sul) are investigating a moderate incidence of oesophageal carcinoma which might be associated with a reputedly common enrichment of wheat flour with *P. canariensis*. Neither the adulteration nor even the commerce in *Phalaris* grain is legally sanctioned, which makes investigation difficult, and the few samples of bread we have examined from this region are apparently free of silica fibres. Silica fibres can be detected in stomach tumours from Japan, where this disease is very common (Henderson et al 1975).

In many parts of the Scottish Highlands (Jarrett et al 1978) cattle suffer from a notably high (25%) incidence of stomach cancer, which has a histological resemblance to oesophageal cancer. This is clearly associated with consumption of bracken. Bracken *(Pteridium acquilinum)* is highly toxic and unpalatable, but when it decays the residue of insoluble detritus among the grass is rich in silica fibres (Parry et al 1985). We have been told that cancer of the eye is common in Queensland among cattle allowed to graze on *Phalaris* when it comes into seed but we have not yet investigated this interesting report.

Opaline silica in human tissue

We have found all these various associations between silica and cancer by examining the environments of people or animals known to exhibit exceptionally high or unusual incidences of cancer. This crude strategy is all too common but is not the way to arrive at any sort of final judgement on the importance of silica in this disease. In an attempt at a more intelligent approach, we have made some studies of silica in human and animal tissue using acid extraction and energy-dispersive X-ray analysis. This has shown that total silica in patients in China is about 10 times higher than in London, but we have not detected any fibres (O'Neill et al 1982). We are far from convinced of

the ability of acid extraction to isolate fibres without breaking them up, or of the relevance of crude estimates of total silica to cancer incidence. Dissolution in acid also prevents us from determining the local effects of silica on the growth of cells *in situ*, which is the only sure way of establishing how it affects the cancer process. The fibres of *Phalaris* are at least 100 times more massive than typical asbestos fibres and hence must be far easier to detect. Nevertheless, even this great advantage has not yet allowed us to find (far less, count) them *in situ* with any confidence. Some sort of detection method is an absolute necessity if we are to undertake the geographical epidemiology and experimental studies which could define the importance of biogenic silica in cancer.

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DISCUSSION

Volcani: What is known about the effects of synthetic fibres of the same sizes as your siliceous fibres?

O'Neill: If you can get the fibres into the pleura, tumours can be induced with alumina or glass fibres. The reason these synthetic fibres do not cause cancer in human may be aerodynamics. Asbestos fibres are much thinner and thus can be inhaled (Selikoff & Lee 1978).

Volcani: I am wondering whether the stimulus is purely a physical irritation, and silicon as such has nothing to do with the effect. I recall that Dr Timbrell at the Medical Research Council Pneumoconiosis Unit in Penarth, Wales, was studying this question. Sincock & Seabright (1975) observed chromosome changes in Chinese hamster cells induced by chrysotile or crocidolite asbestos; fibreglass and glass powder were inert. Hesterberg et al (1982) recently observed that chrysotile, crocidolite and certain fibreglass and quartz dusts induced neoplastic transformation of cultured Syrian hamster cells.

O'Neill: Yes, the stimulus could well be purely physical. It is accepted that non-biogenic mineral fibres can cause karyotypic abnormalities—that is, they can act as mutagens. But we have not yet seen anything of this sort with the biogenic fibres that we study. Nor do they affect the viability of Vero cells, or secretion of growth factors by macrophages (O'Neill et al 1980). Synthetic fibres are very different from these biogenic fibres. Both synthetic fibres and asbestos are extremely heterogeneous in size distribution. The presence of large numbers of smaller particles might account for some of the differences we see.

Last: Animal and epidemiological studies suggest that crystalline silica can cause cancer in the lung; the tumours are epithelial rather than pleural in origin (adenocarcinomas, for example). Silica, as distinct from silicates such as asbestos, seems also to have some sort of specific tumour-causing property, perhaps as a direct carcinogen (that is highly controversial), and as a promoter in the presence of a substance such as benzo[a]pyrene. There seems to be a distinction between the response of the rat and hamster in such studies; there is a peculiar effect of crystalline silica on rat macrophages, which seems not to occur in hamsters. The same process by which crystalline silica does harmful things to macrophages and causes fibrosis, as we discussed earlier (p 190), may have a counterpart in long-term experiments, where silica seems able to cause tumours in rats. And there is very weak epidemiological evidence in humans for an increased incidence of cancer in silicosis patients in Scandinavia, but unfortunately smoking has not been controlled to the point where we can confidently attribute the effect to silica. This is independent of the effects of silicates and asbestos, except where the two might go together; but generally they do not.

O'Neill: There do seem to be two separate mechanisms. Crystalline silica has toxic properties in cell cultures that our biogenic silica fibres have not. I like to think that asbestos works in both ways—the way you have just suggested, and the way that I put forward. That is why it is so effective as a carcinogen. The attractive point about the work on plant silica is that we may be able to isolate a single mechanism for study.

Williams: Is a mineral surface of a fibre more effective in enhancing the spreading of a cell than most organic surfaces, or would this be a feature of any fibre? I can understand why, say, your polyhydroxyethyl methacrylate areas

around the adhesive islands (Fig. 1) do not allow cells to attach, since there is no hydrogen bonding capacity, but what about heavily cross-linked collagen, or cellulose? Are they effective?

Last: Collagen is a superb surface for the spreading of cells, probably the best there is.

Williams: So what direct relevance does silica have to the problem of disease, other than that you happen to find silica fibres?

O'Neill: I have no evidence at all that the chemical nature of silica is relevant. We should mention that you can also produce tumours in rats by implanting sheets of plastic or metals subcutaneously (Brand 1975). The reason why fibres cause cancer is that you are not liable to breathe in a Petri dish, but you could breathe in fibres! Implanted plates of plastics, metals or glass are all equally effective in producing cancer.

Wilson: Tumours can be induced in rats by subcutaneous implantation of discs of the right size of almost any material (Oppenheimer et al 1958). You are suggesting that fibres are essentially promoters. There is a strong association between asbestos exposure and cigarette smoking in cancer production in humans, with a roughly 200-fold increase in lung tumours where the two are combined. Alcohol too has an important effect on oesophageal cancer. Could these be the initiating factors in the populations you were studying, or do you feel that silica fibres are a complete carcinogen, rather than just a promoter?

O'Neill: All the evidence from our animal studies is that biogenic silica fibres act solely as promoters. We therefore have to postulate some additional initiating factor to explain the incidences of oesophageal cancer in humans. Small concentrations of nitrosamines and tar from opium pipes have been identified in Lin Xian and N.E. Iran, respectively, but the concentrations found are far below those which have been used to cause cancer experimentally (Ching et al 1982). However, it must be borne in mind that we still do not know the relative importance of initiation and promotion in human cancer. The human disease can take more than 40 years to develop. Mutations might be accumulating during the whole of that time.

Dobbie: Talc has been detected in gynaecological tumours (Henderson et al 1979). What shape are talc particles?

O'Neill: Talc is often associated with asbestiform minerals and it is difficult to prove that a person exposed to talc has not also been exposed to asbestos particles.

Newman: Talc is basically magnesium silicate but it can be contaminated with tremolite, which is a form of asbestos. In the UK this should no longer happen, with stricter regulation of talc manufacture.

O'Neill: Talc comes in little plates, not very different in size from the islands of adhesive substratum that we used (see Fig. 1). So talc fits into our hypothesis about cell spreading.

Williams: Do I gather that the composition of the fibres is not important, Dr

O'Neill, and that any material will be effective, provided it is of the appropriate size, and persists?

O'Neill: Yes, that certainly sums up what we know so far. However, we have only done tissue culture studies on fibres of *Phalaris* and glass, and animal work with *Phalaris* alone.

Volcani: There are studies on the size distribution of synthetic organic fibres and their effects, and the results are inconsistent. Some fibres will produce proliferative effects and others will not. There is not a perfect correlation.

Birchall: The work of Stanton et al (1977) and others shows that the geometry of the fibre is important and not its chemical constitution. But it has to persist in the body. So if the fibre is made up of a polyaromatic compound and is very stiff, presumably it would give the same problems as an inorganic fibre.

Carlisle: In one study of a large number of fibres that were carcinogenic because of their size and shape, when they were aggregated none of them had any effect, whatever they were made of (Selikoff 1978).

O'Neill: This eliminated fibres over 10 μm in length?

Carlisle: Yes. I would like to add something that may be relevant from our feeding studies in animals (unpublished work, 1985). In one study in rats over many generations, it seemed that the rats on silicon-free diets had a much higher incidence of tumours than the silicon-supplemented animals. In a recent two-year study in rats on low silicon and silicon-supplemented diets, 60% of rats on the low silicon diet had an identical and very large tumour in the brain, so far not characterized, whereas there were no tumours in the animals on silicon-supplemented diet.

Dobbie: Diatoms have siliceous skeletons, as we have heard. Have they been incriminated in the pathogenesis of carcinoma?

Volcani: Not to my knowledge, nor does natural diatomaceous earth, known as diatomite, cause lung fibrosis in humans. Diatom shells (frustule) commonly have no fibres. They vary greatly in shape (e.g. oval, circular, triangular) and in size (2–300 μm).

Dobbie: The finding of diatoms in the lung and bloodstream is used forensically in establishing drowning as a cause of death. If spicules from diatoms gain entry to the body via the gut, is it naive to consider these as a possible carcinogen?

Volcani: The geometry of diatoms doesn't seem to be right; the shells don't have the needle shape which the fibre theory seems to require, and they are too large, as I said.

Werner: This is true for many frustules, but there are diatom species such as many *Chaetoceros* spp. and *Rhizosolenia* spp., abundant in many areas of the oceans, which have very long spines. The spines are longer than 20 μm. Such material could come in contact with humans via products made from infusorial earth.

O'Neill: This is obviously a fascinating area, but the point to make is that we

haven't yet found a fibre of the 'wrong' length (less than 50 μm) that is associated with cancer, or a fibre of the 'right' length (more than 250 μm) that when ingested does not cause cancer, despite much searching. The *Phalaris* fibre is also quite remarkable in its sharpness. The other known fibre that produces cancer is asbestos, which is not pointed but is very narrow, and also is so light that it can penetrate to the bottom of the lung. These seem to be two rather special things. It is not just the presence of a silica particle of about the right size but the question of its entry to the body. Diatoms enter the lung when you breathe in water, but I am not sure that they would enter from the air. I don't know whether siliceous sponges get eaten.

Carlisle: Diatoms have been found in stillborn babies.

Williams: You could perhaps attack these problems by radiocarbon labelling of the hairs, by growing them in ^{14}C-glucose and then doing autoradiography. Carole Perry used this approach when she was following the biosynthesis of the polysaccharides of the cell wall from the silicified macrohairs from *Phalaris canariensis* L.

Perry: The extent of labelling which occurs will depend on when the plant is labelled, as the rate of synthesis of the cell wall and therefore the incorporation of radioactive label varies significantly with the age of the inflorescence. Maximal incorporation of radioactive label into the cell wall occurred at about 14 days after emergence of the inflorescence.

Sangster: Is it possible that the heat from the baking process of the bread in Iran would change the silica fibres chemically?

O'Neill: I don't know. The temperature must reach well over 100°C.

Perry: We have done solid state ^{29}Si NMR experiments of samples of plant silica dried in an oven at 140°C. The spectrum is unchanged from that of the non-heat-treated samples.

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Biocompatibility of silicates for medical use

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Abstract. Implantation of commercial silicate glasses in soft or hard tissue will produce a thick non-adherent capsule consisting of scar tissue. However, special compositions of bioactive silicate glasses and crystallized glass–ceramics bond to bone and soft tissue without such a capsule. Bonding is via ion exchange and formation of active surface layers which incorporate collagen and bone mineral. These bioactive silicates have been studied *in vivo* and *in vitro* and the cellular response appears to depend on total cell surface nectin concentrations as well as on specific nectins and cellular proteins which may be silicon-sensitive. Simple amino acids polymerize and adhere strongly and randomly to binding sites on the bioactive surfaces, in contrast to their epitaxial behaviour on crystalline quartz. Toxicity tests *in vivo* and *in vitro* on powders and on solid forms show no adverse effects associated with bioactive silicates, in contrast with the marked toxicity of crystalline quartz. The bioactivity of the bioactive silicates may be destroyed by addition of small quantities of multivalent ions. The bioactive materials are currently in clinical use and being tested preclinically for a variety of surgical and dental applications.

Implant materials and devices

As many as 40 different materials are used in the 50 different medical and dental devices developed for replacement or repair of tissues or organs of the body (Hench & Ethridge 1982). These biomaterials and devices now form an approximately $3-billion world-wide industry which is expected to double or triple within the next decade (Table 1). Most biomaterials and devices in Table 1 were developed to produce a minimal tissue response, that is to be 'bioinert'. However, it is now well established that no implant material is truly inert in the living body; all materials elicit a response from host tissues. If the material is toxic, the surrounding tissue dies; if the material is non-toxic and dissolves, tissue replaces it; if the material is non-toxic and biologically inactive, a fibrous tissue capsule of variable thickness forms; and if the material is non-toxic and biologically active, an interfacial bond can form between the material and the surrounding tissues (Hench & Wilson 1984).

Silicon-based implant materials can elicit all four types of tissue response:

TABLE 1 Estimated market for biomaterials, USA and total world-wide

Implant	Market in 1985			1995 projections		
	Total no. (millions)/yr	$/implant	Market ($ millions)	Total no. (millions)/yr	$/implant	Market ($ millions)
Intraocular lenses	1.0	400	400	1.5	750	1125
Total hip replacements	0.3	600	180	0.4	700	280
Total knee replacements	0.15	400	60	0.2	500	100
Ankles, elbows, shoulders	0.05	500	25	0.7	600	420
Finger joints	0.4	50	20	0.6	100	60
Mammary prostheses	0.4	100	40	0.5	120	60
Tooth implants	0.3	500	150	1.0	300	300
Dental ridge augmentation; periodontal materials	0.2	20	4	2.0	40	80
Cardiovascular (heart pacers, valves, by-passes, arterial prostheses)	1.0	250	250	1.5	300	450
Middle ear prostheses	0.03	150	4.5	0.035	200	7
Cochlear prostheses	0.0005	20 000	10	0.01	10 000	100
Orthopaedic fixation devices	1.0	50	50	1.2	60	72
General (sutures, skin grafts, catheters)	2.0	20	40	3.0	25	75
Total (US)	6.8	—	1233	12.6	—	3129
Total (world-wide)	12.0	—	2800	25.0	—	6000

toxic, degradable, 'inert', and bioactive. This article discusses: (1) the chemical and physical factors responsible for the variety of tissue responses to silicon-based implants; (2) the toxicity tests necessary to establish the safety of silicon-based materials for prosthetic devices; (3) potential medical and dental applications of bioactive silicates; and (4) the biological factors responsible for bioactive versus bioinert responses to silicates.

Bioactive silicates

The concept that a manufactured material could be formulated to form a chemical bond to living tissues was first proposed by one of us (L.L. Hench) to the US Army Medical R & D Command in 1967. In November 1969, results of experiments on a series of silicate glasses that were discovered to bond to living bone were reported (Hench et al 1971). These glasses, containing (by weight) 45–55 % SiO_2, 12–24 % Na_2O, 12–24 % CaO, and 3–6 % P_2O_5, are now called Bioglasses® (® registered trademark, University of Florida, Gainesville, FL 32610) to signify 'special glasses designed to elicit a specific biological response by means of controlled surface reactions.'

Fig. 1, region A, summarizes the compositional dependence of the Bioglass® bone-bonding boundary. (Note that this diagram is for glasses with a constant 6 % P_2O_5 content by weight.) Silicate glasses within region B (such as window or bottle glass or microscope slides) behave as 'bioinert' materials and elicit a non-adherent fibrous capsule at the implant–tissue interface. Silicate glasses within composition range C are resorbable and have been removed from the
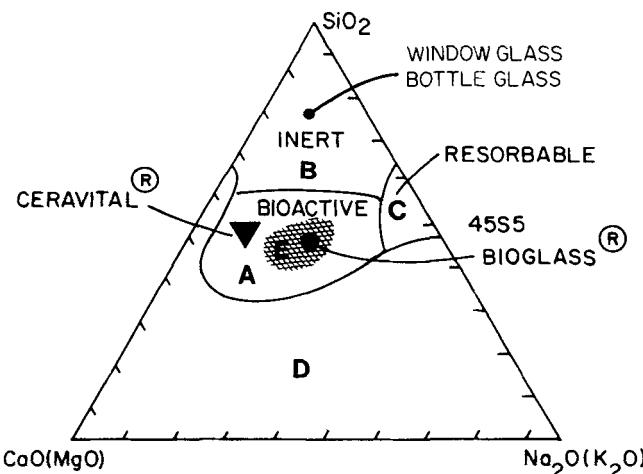
FIG. 1. Diagram showing the compositions of Bioglass® and other glasses.

implant site after 10 days to 30 days' implantation. Glasses within region D are not technically useful so have not been tested biologically.

Studies on soft tissue responses to bioactive silicates (Wilson et al 1981) indicate that the collagenous constituent of soft tissues can strongly adhere to bioactive silicate glasses which lie within the compositional range shown in Fig. 1 as region E.

Many bioactive silicate glasses which have been investigated in detail are based on the formula called '45S5', signifying 45% SiO_2 by weight (the network former) and a 5:1 molar ratio of CaO to P_2O_5. Glasses with lower molar ratios of CaO:P_2O_5 do not bond to bone. However, substitutions in the 45S5 formula of 5 to 15% B_2O_3 for SiO_2, or 12.5% CaF_2 by weight for CaO, or replacement of Na_2O with K_2O, still allow bone bonding. Crystallization of the various Bioglass® compositions to form glass–ceramics (Hench et al 1971) has no measurable effect on the ability of the material to form a bone bond. However, addition of as little as 3% Al_2O_3 by weight in the 45S5 formula prevents bonding (Hench & Ethridge 1982).

Gross & Strunz (1982) have shown that a range of low-alkali (0 to 5% by weight) bioactive silicate glass–ceramics, termed Ceravital® (® registered trademark, Leitz Co.) also bond to bone. They find that small additions of Al_2O_3, Ta_2O_5, TiO_2, Sb_2O_3 or ZrO_2 inhibit bone bonding. A two-phase silicate–phosphate glass–ceramic composed of apatite [Ca_{10} ($PO_4)_6$ (O_1F_2)] and wollastonite [CaO·SiO_2] crystals and a residual silicate glassy matrix, termed A/W glass–ceramic by the Kyoto University development team (Nakamura et al 1985), also bonds with bone. Addition of Al_2O_3 or TiO_2 to the A/W glass–ceramic also inhibits bone bonding whereas incorporation of a second phosphate phase, B-whitlockite (3CaO·P_2O_5), does not.

Another multiphase bioactive phospho-silicate (Höland et al 1983) containing phlogopite, (Na,K) Mg_3[$AlSi_3O_{10}$]F_2, and apatite crystals bonds to bone even though Al_2O_3 is present in the composition. However, the Al^{3+} ions are incorporated within the crystal phase and presumably do not alter the surface reaction kinetics of the material.

Silicate surface reactions

The surface chemistry of bioactive silicates is best understood in terms of six possible types of surface reactions (Hench 1982). As shown schematically in Fig. 2, a silicate may react with its environment only by developing a surface hydration layer. This is called a type I response. Vitreous silica (SiO_2) or crystalline quartz behaves in this manner when exposed to a physiological environment, as do inert glasses which fall into the apical area in region B of Fig. 1.

When sufficient SiO_2 is present in the glass network, the surface layer that forms from alkali–proton exchange can repolymerize into a dense SiO_2-rich
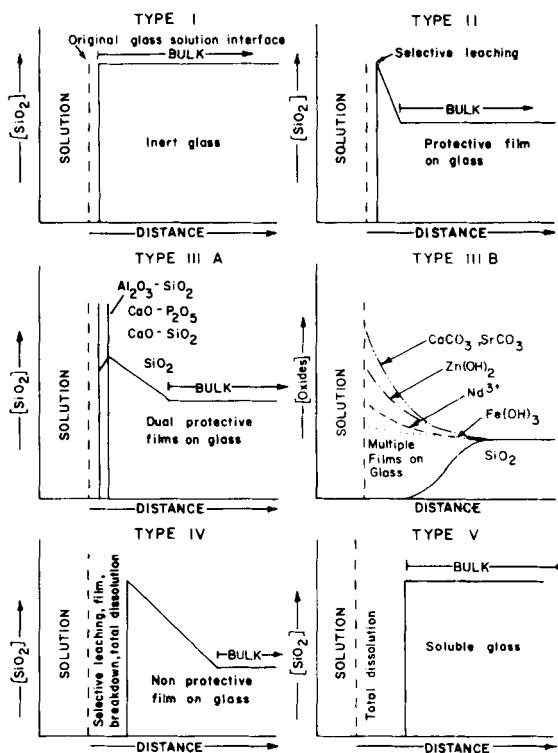
FIG. 2. Types of surface reactions of glasses and silicates.

film which protects the glass from further attack. This type II surface is characteristic of most commercial silicate glasses, and their biological response is typical of glasses within the remainder of region B in Fig. 1.

At the other extreme of the reactivity range, a silicate glass or crystal may undergo rapid ($r = k_1 t^{0.5}$) selective ion exchange of alkali ions with protons or hydronium ions, leaving a thick but highly porous and non-protective SiO_2-rich film on the surface—a type IV surface. Under static or slow flow conditions the local pH becomes sufficiently alkaline (pH>9) for the surface silica layer to dissolve at a rate of $r = k_2 t^{1.0}$, leading to uniform bulk network or stoichiometric dissolution—a type V surface. Both type IV and V surfaces fall into region C of Fig. 1.

Type IIIA surfaces are characteristic of bioactive silicates. A dual protective film rich in CaO and P_2O_5 forms on top of the alkali-depleted SiO_2-rich film. When multivalent cations such as Al^{3+}, Fe^{3+} or Ti^{4+} are present in the glass or in the solution, multiple layers form on the glass as the saturation of each cationic complex is exceeded, resulting in a type IIIB surface, which does not bond to tissues.

Bonding mechanisms

The tissue-bonding mechanisms of bioactive silicates involve a complex series of physiochemical and ultrastructural events (Hench & Clark 1982, Hench 1981). These steps include: (1) rapid exchange of Na^+ or K^+ with H^+ or H_3O^+ from solution; (2) loss of soluble silica to the solution, due to breaking of Si–O–Si bonds, and formation of Si–OH and $Si(OH)_4$; (3) condensation and repolymerization of a SiO_2-rich layer on the surface; (4) migration of Ca^{2+} and PO_4^{3-} groups to the surface through the SiO_2-rich layer; (5) formation of a $CaO–P_2O_5$-rich film on top of the SiO_2-rich layer; (6) growth of the SiO_2-rich layer by diffusion-controlled alkali ion exchange; (7) growth of the amorphous $CaO–P_2O_5$-rich film by incorporation of soluble calcium phosphates from solution; (8) crystallization of the amorphous $CaO–P_2O_5$ film by incorporation of OH^-, CO_3^{2-} or F^- anions from solution to form a mixed hydroxyl, carbonate, fluorapatite layer; (9) agglomeration and chemical bonding of the apatite crystallites around collagen fibrils and within adsorbed mucopolysaccharides (glycosaminoglycans) and other proteins produced by osteoblasts or fibroblasts.

   The consequence of this sequence of reactions is the creation of an interfacial bonding zone between tissue and the implant that consists of a 30 μm layer of $CaO–P_2O_5$ on top of a 100–120 μm layer rich in SiO_2. Fig. 3 shows the direct bonded interface between rat tibial bone and a 45S5 Bioglass® implant 30 days after implantation.

   Steps 1–5 occur within minutes of implantation, thereby disguising the silicate implant to the physiological environment as if it were mineralizing apatite. Steps 6–9 require 7–10 days to complete and are controlled by the type and concentration of stem cells at the implant interface. The ratio of silica in the glass to alkali and alkaline earth ions ($SiO_2/R_2O + RO$) determines the rate of formation and thickness of the bonding zone. As the bone-bonding boundary (Fig. 1, region A) is approached the rate and thickness decrease.

   In the presence of osteogenic precursors, the bioactive silicates favour formation of osteoblasts which govern the further steps of bone development (Hench & Ethridge 1982). Gross & Strunz (1982) summarize their ultrastructural findings on the bioactive silicate bone-bonding process as follows: 'Within the extracellular matrix and between small bundles of fibrils, matrix vesicles appear and display small, electron-dense, needle-like crystallites assumed to be apatite. After rupture of the vesicle membrane, calcifying fronts are formed. Often this process begins and is therefore more pronounced in the surroundings of the implant and the adjacent osteoblast, but may also start in the area around the osteoblast and then involve the surroundings of the already mineralized seam of amorphous cementing substance at the interface. Later on the whole area is mineralized, the osteocytes being rather evenly distributed and often arranged with their long axes parallel to the surface of the implant.
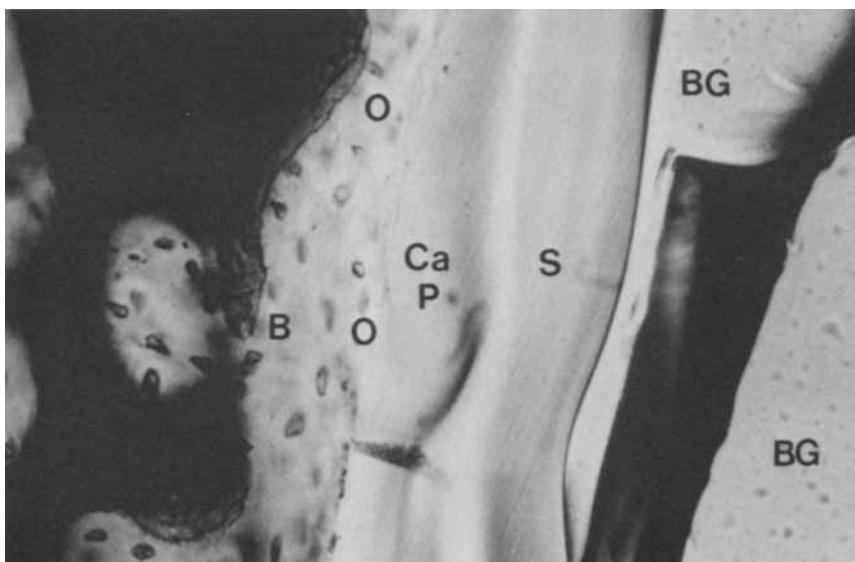
FIG. 3. The interface between rat tibial bone and a Bioglass® implant, 30 days after implantation. B, bone; O, osteoblasts; CaP, calcium/phosphorus-rich layer; S, silica-rich layer; BG, Bioglass®. Magnification × 200.

This feature is found in different animal species—rats, dogs, pigs, chickens and humans—and provides the morphologic basis for the biomechanical quality of the bone bonding.' Gross & Strunz (1982) have also shown that addition of the multivalent cations Al^{3+}, Ta^{5+}, Ti^{4+}, Sb^{3+} or Zr^{4+} inhibits bonding. In all these models unmineralized osteoid and chondroid persist at the implant interface, indicating the presence of substances that impede mineralization or the absence of substances that promote it. The inhibited cells at these interfaces do not switch from the production of metachromatic ground substance and type II collagen to the production of type I collagen and organelles for mineralization. In contrast, bioactive silicates with less than critical concentrations of such multivalent cations show bonding with osteoblasts and produce matrix vesicles, and normal mineralization proceeds at the interface. Bonding silicates release monophosphates at their interface, whereas non-bonding compositions release tri-, tetra- or polyphosphates. Local phosphatase concentrations and the formation or function of matrix vesicles can be altered by the local monophosphate: polyphosphate ratios and mineralization is thereby inhibited or enhanced.

Interfacial concentrations of soluble Ca and Si are also strongly influenced by silicate composition. Multivalent cations, which convert type IIIA bonding surfaces to type IIIB non-bonding surfaces, inhibit release of soluble Ca and Si.

The concentration and function of osteocalcin, Gla (γ-carboxy glutamic acid) proteins, osteonectin, and other extracellular glycoproteins at the implant interface may well be controlled by soluble Ca or Si concentrations and this type of molecular complexation.

Toxicity tests

Safety has been one of the most important questions addressed during the studies of bioactive silicates. This is because early development of implant materials had emphasized 'bioinertness' and minimal encapsulation by fibrous tissue. The time-dependent control of interfacial tissue bonding which is characteristic of bioactive implants required new thinking on appropriate toxicity tests for implant materials, and proof of non-toxicity in a wide range of tests was needed, including those appropriate to particles of the material, should they be produced by abrasion or wear during use. A total of 17 *in vitro* and *in vivo* tests, conducted on 45S5 Bioglass® and related compositions, were summarized by Wilson et al (1981). All tests showed the bioactive glasses to be non-toxic and biocompatible. Even one of the more sensitive *in vitro* tests, ingestion of particles by macrophages (Pigott & Ishmael 1979), showed virtually no toxicity. Seventy-two hours after exposure to powder in culture, silica from the glass was detectable by analytical transmission electron microscopy within an apparently viable cell, in marked contrast to crystalline silicates which are immediately toxic to such cells. The lack of toxicity of bioactive silicates has been confirmed in numerous laboratories in the US, East and West Germany, Italy, Belgium, France, Denmark, and Japan.

Applications

The most obvious applications for devices made from bioactive glass and glass–ceramics are in the replacement of hard tissues (Hench & Ethridge 1982, Hench & Wilson 1984). Limitations are only on mechanical strength, which is not sufficient for load-bearing devices; however, their machinability makes them ideal for applications as middle ear devices (already available), dental root replacement, and maxillofacial augmentation and repair. Glass–ceramics are also used in these applications, although the transparency of glass and more rapid machining under surgical conditions offer an advantage under certain circumstances. For load-bearing devices such as single tooth implants, orthodontic anchors and joint replacement, the strength of metals or inert ceramics can be combined with the bonding ability of the bioactive glass used as a coating on stainless steel, titanium or alumina, so allowing chemical, rather than cement or mechanical, fixation of devices.

In powder form the bioactive materials may be used as a treatment for bone loss in periodontal and other dental diseases, and for maxillofacial augmentation.

Development of these materials in combination with metal fibres, polymers or collagen will provide composite materials with the correct combination of properties to allow their use in plastic surgery and orthopaedic surgery.

Silica versus silicates in biology

The contrast between the biological responses of crystalline SiO_2 (quartz) and the bioactive silicates is one of the most dramatic in nature. Quartz elicits a thick, non-adherent fibrous capsule when implanted in bulk form, and a marked toxic reaction when present in the body as particulates. Bioactive silicates are non-toxic as particulates and in bulk can form a strong interfacial bond with both hard and soft tissues. What is the origin of these differences? What are the biological consequences?

As reviewed above, quartz behaves as a type I surface with only surface hydroxylation. It has a very low isoelectric point (pI) at pH 2.2 and therefore presents a very acidic surface to tissues and metabolites. In contrast, bioactive silicates exhibit a kinetic, type IIIA surface which changes its pI as a function of time from pH 2.4 to pH 8 to 9. This is due to formation of a biologically active apatite film and a localized alkaline buffering of the interface (Hench 1981). Thus, the bioactive silicates provide a broad range of positive and negative bonding sites which change with time. They also provide a high density of such surface bond sites and therefore can attach to a great variety of metabolites which appear in the physiological milieu during wound healing (Hench & Paschall 1973).

Some *in vitro* experiments illustrate the vital differences between these related but vastly different silicon-based materials. Hartwig & Hench (1972) compared the epitaxial polymerization of poly-L-alanine and poly-D-alanine on single crystal quartz and 45S5 Bioglass® substrates. The effects of crystal orientation, radio frequency (r.f.) plasma cleaning, and adsorbed gas layers on the orientation and form of the polyamino acids were measured quantitatively, using scanning electron microscopy, and interfacial bond strengths were assessed qualitatively. The deposition method used was equivalent to Seifert's method (1966) and the results were compared with those calculated from Seifert's photomicrographs.

The sheaf-like aggregates of poly-L-alanine were oriented on the (1010) face of laevorotatory quartz at +45° to the axis (Fig. 4A). This orientation corresponds with the $[01\bar{1}1]$ direction on the $(10\bar{1}0)$ quartz surface. The SiO_4^{4-} structural units in this direction have successive repeats of 0.72 nm, which is the repeat distance of the primary polypeptide conformation of poly-L-alanine.

Poly-L-alanine deposited on the $(10\bar{1}1)$ crystal face of quartz oriented epitaxially at 35° and 70°, which also matches the SiO_4^{4-} repeat distances and polypeptide conformational repeats (Fig. 4B).

Most importantly, dextrorotatory poly-D-alanine showed no epitaxial
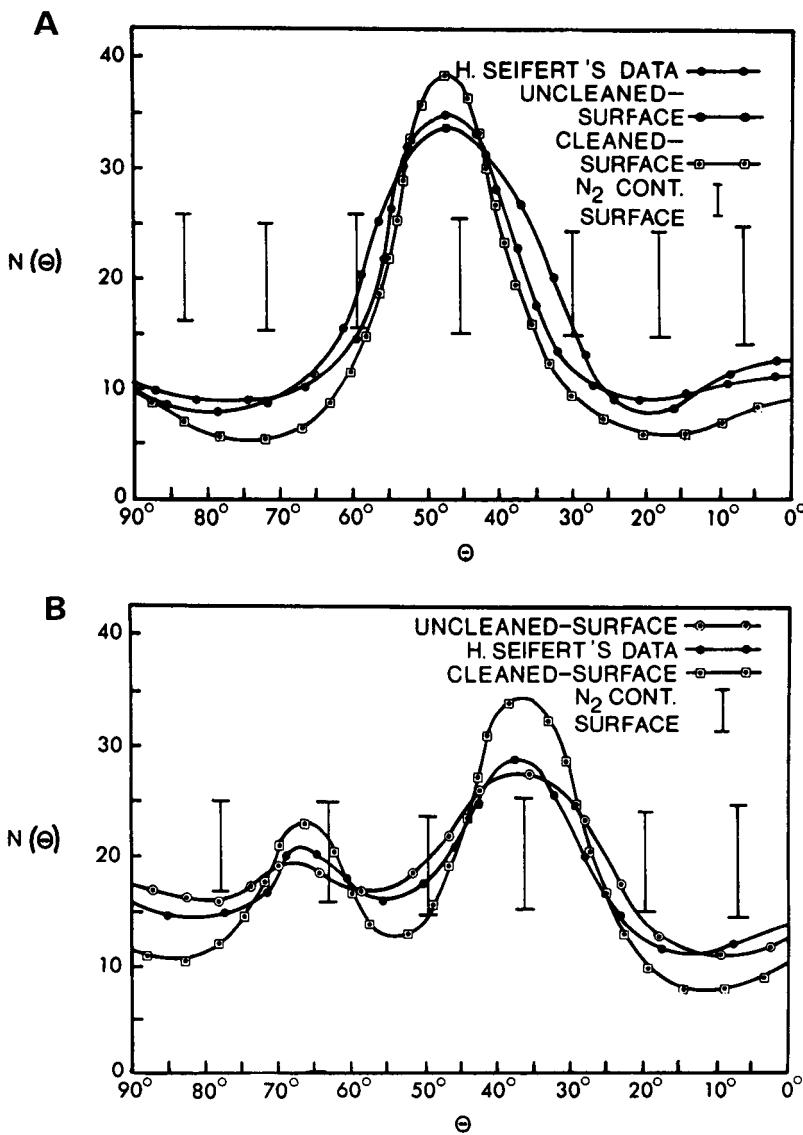
FIG. 4.   Polymerization of poly-L-alanine on (a) the (10$\bar{1}$0) prism face of laevorotatory quartz, and (b) on the (10$\bar{1}$1) prism face.

orientation on either quartz surface, confirming the necessity for optic axis coincidence. The amorphous 45S5 Bioglass® substrate also did not induce a preferred orientation of either the polymerized L or D amino acids.

Exposure of the quartz surfaces to r.f. plasma before deposition of the poly-L-alanine increased the epitaxy (Fig. 4A,B) due to removal of surface contaminants and an increase in active silanol sites. Adsorption of moisture on the plasma-cleaned surface enhanced epitaxy even more. However, adsorption of N_2 on the plasma-treated surface destroyed the epitaxy of the amino acid completely.

The significant differences between the bioactive glass and quartz were in the randomness and adherence of the polymer. Unoriented poly-L-alanine deposits were easily washed from the surface of the quartz crystals, whereas the oriented epitaxial polymers were so tightly adherent that they could only be removed by grinding. Poly-D-alanine washed off with no adherence. In contrast, poly-L-alanine attached to the Bioglass® surface as a very dense but unoriented deposit. The adherence of the random amino acid deposits on the bioactive glass was equivalent to that of the best of the oriented deposits on the quartz surfaces.

Strong, irreversible, attachment of amino acids and other metabolic precursors in dense, random configurations on specific glass compositions may have served a vital function in the origination of precytic complexes (precytes are cell-like structures arising through the self-organization of completely or partly abiotically formed constituents). Schwemmler (1984) cites the necessity of inorganic intermediaries for precyte evolution. The specific orientational epitaxy of simple amino acids on quartz and the dense random adherence of pre-metabolites on volcanic glasses exhibiting type IIIA behaviour could certainly serve as the irreversible, bioactive substrate interactions required for precellular and cellular chemical evolutionary processes.

Additional important evidence for the unique behaviour of bioactive silicates in altering the function of cells was reported by Seitz et al (1982). It was discovered for the first time that cells could be retained in a prolonged resting state when grown on a bioactive silicate surface (45S5 and 52S4.6 Bioglass® substrates); that is, they did not spread and become confluent. This behaviour was characteristic of both NIL cells (a fibroblast type with surface fibronectin) and CHO (fibroblasts without surface fibronectin) cell lines. The length of the prolonged resting state was several days for the NIL line and more than a week for the CHO line. When 45SF Bioglass® substrates were pre-treated with fibronectin, both types of cells attached, spread and became mitotic at the same rate as on the more usual inert type II soda–lime–silica glass substrates. The prolonged resting state did not produce any irreversible change in the cells, which resumed division and spread normally when removed and seeded onto inert substrates. S. Ito at Kagoshima University (1985, personal communication) and U.M. Gross at the Free University of Berlin (1985, personal communication) have also observed prolonged resting states for other fibroblast cell lines (L cells and HeLa cells) maintained on bioactive silicate surfaces.
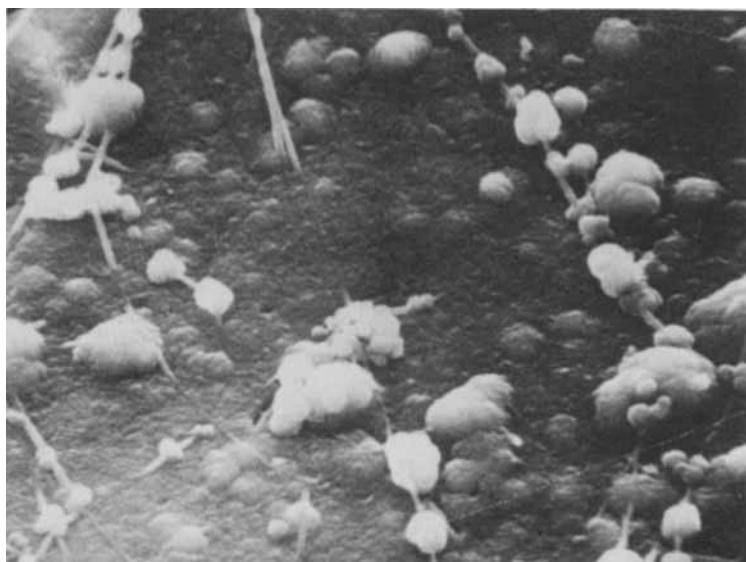
FIG. 5. The incorporation of collagen and apatite in a Bioglass® surface at 37 °C *in vitro*. Magnification × 5000. (Figure by courtesy of C. Pantano.)

In contrast to the fibroblast lines, Hench & Ethridge (1982) report work by R. Grant that showed rapid attachment, spreading and mitosis of osteoblast-like primary cultures on 45S5 Bioglass® substrates. There was no noticeable difference in the behaviour of the osteoblast-like cells on inert or bioactive glasses. These findings have led to a general theory (see Chapter 14 of Hench & Ethridge 1982) that the bone bonding of bioactive silicates is due to the establishment of a competitive advantage for osteogenic precursor cells compared to fibroblasts. It is suggested that this is due to the presence of biologically active silanol, calcium and phosphate groups on the type IIIA bioactive silicate surfaces. These bonding sites presumably match cationic-specific nectin attachment complexes on progenitor cells. These may then activate the cell membrane receptors that are involved in the process of differentiation into osteoblasts, which have matrix vesicles and can produce type I collagen. Types I, II and IIIB silicate surfaces do not develop these cation-specific membrane attachments and non-specific fibroblasts proliferate unimpeded.

The surface reactions of bioactive silicates can have major effects on other biological interactions. For example, an *in vitro* model where collagen fibrils in solution are exposed at 37 °C to a 45S5 Bioglass® substrate shows (Fig. 5) that the apatite film growing on the substrate forms agglomerates around and bonds to the collagen, and incorporates the fibres into the growing surface layer. This

interdigitated organic–inorganic structure is equivalent to that formed in living systems when materials join with disparate elastic moduli, such as Sharpey's fibres which bond teeth into the periodontal membrane, or tendons and ligaments bonding to bone. The ultrastructural features demonstrated in Fig. 5 are a result of physical chemical reactions alone; no cells were present in this model. Addition of a mucopolysaccharide (glycosaminoglycan) such as chondroitin-D sulphate to the *in vitro* Bioglass®–collagen solution produces an even denser, more highly organized organic–inorganic interface.

There is growing evidence that the enormous variation in surface chemistry of sparingly soluble silicates can play a major role in altering and/or controlling biological interfaces with such substances. Interfacial bonding of both bone and soft tissues with bioactive silicate implants is one of the immediate applications of this unique surface chemistry. However, understanding the effects of inorganic substrates in cellular evolution and differentiation may be an even more important long-term scientific consequence.

Acknowledgements

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DISCUSSION

Birchall: Do all your bioactive silicate glasses contain phosphorus?

Hench: Yes. We found that if we eliminate phosphorus and stay in the right compositional range, the surface of the implant collects phosphorus from the body fluids.

Birchall: So the bonding of collagen is always through calcium phosphate?

Hench: It seems to be.

Williams: To what extent do you think mobility in the matrix of the silicate implant is important? You say that phosphate acquired from outside the implant will be sufficient. It seems to me that it won't. Do you not need the restructuring of the surface in order for it to become in some way compatible with calcium phosphate?

Hench: If we eliminate phosphate from the glass, bonding occurs but it is much slower and it takes longer for the interfacial bond to mature. If phosphate comes from the inside of the implant, the phosphate seems to be providing the nucleation for calcium phosphate on the implant which allows hydroxyapatite mineralization to happen more quickly. At present there are therefore no bioactive materials in use or under exploration that do not include phosphate.

Williams: The question is whether diffusion of ions within the glass is an essential feature. If it is, then ion diffusion can be controlled in well known ways. For example you can regulate it by the way you choose the mixtures of cations in the glass, and you are not restricted to sodium and potassium ions, although they are the cheapest to use. You could also use lithium, caesium, barium or strontium, and so on. As you adjust ion composition by changing ion size, the ion mobilities in a silicate lattice or in glasses are adjusted. The rates of the 'skin formation' on the surface of the mineral can therefore be controlled by the chosen mixture of the components. This could be very important if the surface has to re-form to achieve biocompatibility. I ask if this is the case for your materials since this could explain why they are so harmless compared with asbestos. In a material like asbestos the migration of ions is extremely slow because of the higher charges of the cations. Perhaps one of the problems with

harmful silicates is that they cannot restructure and therefore fail to become biocompatible.

Hench: We have seen a critical requirement of a polymerizing silica layer on these materials for bonding; that extends to the German and Japanese studies also, as cited in our paper. The problem with making that a requirement and not just a potential side-effect is that dense apatite implants also form bone bonds. The mechanism seems to be different; it appears to be a nucleation of biological apatites at the surface, without requiring the silica condensation first. There is still the possibility that the sparingly soluble concentrations of calcium phosphates released from these apatites are complexing with silicon from the body fluids and form the precursor interface. An indication that this might be happening is that titanium implants take a long maturation period for bonding, of many weeks. When the implants are examined as they have begun to develop the bond, silicon concentrations have been seen on the surface of the titanium. Thus, we don't know whether the polymerizing of silica is critical in formation of tissue bonds to implants, but it looks as if it may be.

Williams: So there is a diffusion mechanism from the materials of the body, but in addition there is a diffusion mechanism from the implanted material. Dr Mann said, concerning biogenic silica, that $Si(OH)_4$ could be lost very easily by diffusion from the central part of silica rods. This could make them unharmful in humans. There are also the diffusive mechanisms for getting electrical effects in solids and so on. These diffusive mechanisms are important in the properties of many biological solids.

One last point: if you now put a trivalent ion into the biosilicate does this wreck the biocompatibility?

Hench: Yes!

Williams: The trivalent ion will ruin all the internal diffusive possibilities, because it binds the system too tightly. Compare for example asbestos.

Birchall: You would not need to put it in the glass. Al^{3+} adsorbing on the silica-rich layer from the aqueous side would ruin the adsorption of protein. This is a nice example of the opposite effects of Al and Si.

Wilson: Some of the bioactive glass coating studies were done on alumina ceramic (see p 238), and the problem in the early stages was to achieve a coating without getting the aluminium ions into the glass.

Werner: What happens if you include borate in the system, by comparison with phosphate?

Hench: We have substituted for silica with up to 15% of B_2O_3 in the bio-active glass composition and it still bonds to bone. We haven't gone beyond that amount.

Werner: Would this improve the quality of the glass?

Hench: No, it doesn't. This is far outside the Pyrex range of compositions; it is a bad glass for controlling physical properties.

Volcani: I am curious about the bonding between polyalanine and the

Bioglass surface. Is that hydrogen bonding, since polyalanine is essentially neutral? Did you try polylysine, for example, which is positively charged?

Hench: I don't know what the bonding mechanism is. We tried one other amino acid, glutamic acid, but the results were difficult to control. We didn't see any preferred epitaxial polymerization, however.

Volcani: Is the surface of Bioglass negative in charge?

Hench: It is initially negatively charged and then becomes positively charged as the apatite layer forms. In contrast, quartz is always negatively charged.

Volcani: Polylysine might then be more effective than polyalanine at bonding, since it would neutralize the charge of the Bioglass surface.

Dobbie: Have you tested the reaction of Bioglass with skin?

Wilson: We haven't done so yet. It is significant in applications such as transcutaneous leads and dental implants; you can successfully prevent epithelium growing down around an implant if you have contact between the collagen fibres of the subcutis and the implant, below the epithelial layer. We believe that Bioglass implants will work transcutaneously even if they don't interact positively with epithelium, although we have no reason to doubt that they will.

Dobbie: Would the mechanical properties of the Bioglass allow it to be cast into a structure which would be compatible with sitting in the skin?

Wilson: It would very much depend on the biomechanics of the system, how much and how often movement occurred at the interface of implant and tissues due to applied forces.

Hench: Ideally, we need a much more elastically compliant transcutaneous device with the Bioglass coating to satisfy biomechanical requirements.

Dobbie: Do you think Bioglass is so successful in a biological situation because the only transepithelial objects which pass through a body surface are teeth and they also possess an apatite coating?

Wilson: Yes, this is one of the areas we are now working on.

Hench: The histology of the Bioglass implant at the transcutaneous interface looks very much like that of a natural tooth.

Wilson: Showing slides of the interaction *in vitro* with collagen (e.g. Fig. 5) to dentists is interesting, because they immediately recognize the similarity to the appearance of the surface of an extracted tooth.

Final general discussion

Williams: The solution chemistry of silicon seems to be not too well formulated at the moment, seemingly because the chemistry is very difficult. How should we be tackling chemical speciation? Which chemical species are we talking about in biological solutions, and do we need new methods for analysing solutions?

In contrast with what I have just said about solutions, a lot of minerals are extremely well characterized, even though they are of variable composition. It is with minerals approaching silica gels or those like glasses, which have migratory properties, that there is great difficulty in talking about speciation because there is no uniformity of composition within the substance. In the silica minerals in biology we don't know the nature of the Si–OH bond or how it varies along the surface or through the bulk. There is also the problem of the time-dependence of the system. Maybe there is no simple chemical definition of these systems.

The physical properties of these biominerals depend on the way in which the composition changes. If the composition is not regular, there is no isotropic physical property or even a regular anisotropic physical property except locally. Defined properties will therefore be extremely hard to describe, partly because of migrations of species within the matrices in different places and then a build-up of a new matrix within the species. We have a grave problem in handling the description of the amorphous biominerals.

We discussed also the molecular interactions in solution with organic molecules but we don't seem to have defined a single biological organic molecule, which means we are in great chemical difficulty. This leads back to the problem of the biominerals, which is one problem which everyone is trying to solve. We have not been able to describe the interface with biominerals because we don't know the organic or the inorganic side, except in a very rough and ready way.

Werner: How is the arrow from minerals to solutions and back (Fig. 1, p 2) affected in the soil by plant roots with a different uptake system or exclusion system for silica in solution? We know that there are accumulators (grasses) and non-accumulators (legumes) for silica in plants. It would be very interesting to study systematically the dissolution from silica minerals as affected by plant roots with different rates of silica uptake, in the presence and in the absence of other components of plant nutrient solutions.

Hench: Reaching a thorough understanding of the rates of reaction is difficult because surface interface replenishment influences mineral leaching very

strongly. Variables such as the ratio of surface area to volume of contacting solutions, relative flow rates and the depletion of species by sorption, all affect time-dependent concentrations of species and the fluctuations of concentration (Hench 1982). This can shift the controlling rate of reaction from one species to the rate of another species, with time, and then vice versa. This means there are rate equations with rate constants for every species, each of which is concentration-dependent, but the concentration dependency is time-dependent. In the end, rate equations usually are empirically based. This is critical for nuclear waste. People have almost given up hope of being able to establish a thorough thermodynamic foundation for the rate equations.

Williams: Are there technical methods of studying these types of material which are not being used or have not been described here?

Hench: No one here has presented a simple model system for answering specific questions involving the interface between minerals and biological systems. It may not be possible to find one.

Farmer: There are examples of lower species of plant life mediating the dissolution of minerals. Jones & Wilson (1985) have shown that some lichens growing on rocks produce oxalates; magnesium oxalate was identified under lichens growing on magnesium-rich rock (serpentine) and calcium oxalate in lichen on calcium felspar.

It has also been shown (Duff et al 1963) that certain bacteria produce 2-oxogluconic acid, a complexing acid which dissolves phosphate and silicate minerals in *in vitro* cultures. One can be sure that the higher plants, too, exude complexing acids which will attack minerals in the root zone and bring them into solution. The primary rock minerals cannot be regenerated in the soil environment. Crystallization of secondary aluminosilicates, such as kaolinite, is too slow to trap silicon from solution.

Williams: You described what happened with calcium and aluminium. Has silica appeared in any of those plant experiments?

Farmer: Silicon is seldom left as a silica gel in mineral weathering. It is usually liberated into solution. In a neutral soil, it then has only two places to go. Either it is taken up by plants and recycled as phytoliths or it is washed out of the soil. The overall balance is that what comes out at the bottom of the profile must, in a steady state, equal the rate of mineral weathering. In podzols, some silicon can be trapped in aluminosilicate precipitates (allophane and imogolite). In arid regions, opaline silica can be deposited from solution.

Williams: And that is true for the lichens as well, on a silica-based rock?

Farmer: Wilson et al (1981) found a fibrous amorphous silica gel as a weathering residue of serpentine, under lichens.

Volcani: Various microorganisms (bacteria, actinomyceles, fungi, algae and lichens) participate in the dissolution of silicate rocks and minerals by excreting organic acids (e.g. acetic, formic, oxalic, fulvic and humic acids), inorganic acid (sulphuric) and alkaline products (ammonia, amines). Chelating agents are

also implicated in the weathering of rocks (for reviews see Silverman 1979, Ehrlich 1981, Krumbein & Werner 1983).

Williams: So you would say that there is no mechanism by which a biological system can attack quartz directly, even by making the surface alkaline?

Volcani: Not to my knowledge.

Birchall: In binoculars used in jungles during World War II some bug reduced the clarity of the lens by etching it. I don't know what the bug was but the silicate in the glass was dissolved.

Williams: But again no organic-mediated process was discovered in any of these systems. There seems to be a renewal of the metal ions before the silica dissolves. No simple organism we know of can handle silica in the way that organisms can handle calcium carbonate or oxalate.

Sullivan: What happened to the report of a silicase enzyme which liberates silica from pectins, connective tissue and so forth?

Carlisle: Silicase was postulated to be present in the intestinal tract by Schwarz (1978). Hasn't some organism called *Bacillus siliceus* been reported?

Volcani: Not that I know of. I only know of the organisms that can break up silicates.

Williams: But none of those to your knowledge provide an organically created silica mineral?

Volcani: No.

Birchall: Schwarz's experiment (1978) in which he thought he had an enzyme was based on hydrolysis of an artificial long chain fatty silicate. A simple pH dependence could be misleading in such an experiment.

Werner: In Fe nutrition in plants and in bacteria there are siderophores known with very high Fe-binding affinities. For silicic acid there may be no need for similar compounds in the soil, since the concentration of silica in the soil may be high enough in most cases to supply the demand for Si as an essential trace element. But this is an assumption.

Sullivan: Binding proteins for sulphate have been isolated and identified and specific ionophores for iron are very well studied.

Sangster: I believe that humic acid in organic soils is reported to increase the availability of silica for plant uptake. Could someone comment on the reason for this?

Hench: Has anyone any knowledge of an alkali contained in rocks?

Richards: We have made a film of living fibroblasts treated with white asbestos (magnesium silicate). Before the cells are treated we see undulating membrane movement. After a massive dose of asbestos there are only a few cells and many thousands of particles. All the undulating membrane activity seems to stop immediately but before anything goes into it the cell cringes as if something had hit it. All the contacts the cells keep with their neighbours start to disappear. There are still some areas with a little bit of membrane movement and we can see what could be phagocytosis. In the electron microscope that

area doesn't seem to be a proper membrane. I think there is something about the chemistry of that area of the surface and what is around the fibres that allows them to go in at certain points on this cell. I can't explain it exactly except that all these particles which now build up seemingly inside the cell do not seem to be in a membrane-bound vesicle. The cell can take in an enormous amount of material and still survive, though it may have an indigestion problem.

We also gave normal cells a treatment with another particulate substance which induces cell death very nicely. Cell death is a very slow event. After treatment these cells commit suicide. There is a stringent pulling-out effect. The membrane tends to stop working, the cells cringe in, and then blow up. The blow-up occurs by a sort of volcanic activity over quite a long period of time. The cell doesn't just burst and die but does it very gradually. The explosions occur on the surface.

To me the surface chemistry of that particle really decides how it is processed and how it has a membrane effect. I see another layer—another biological chemistry, if you like—between magnesium silicate and the membrane of the cell—which determines recognition of the 'particle'. Every particle has its own biological coat and the manner in which it exchanges components of this coat determines how it will react with membranes and internal organelles once phagocytosed. [Illustrated with a cine film.]

Williams: That brings us directly to the question of speciation of fibres. Speciation is said to be both chemical and physical and that is pretty well agreed. There is a certain restriction on the size of fibres and an unknown chemical factor, which together produce the observed biological effects.

Richards: We need the chemists to give us some absolutely defined chemical surfaces and keep everything that we are dealing with to exactly the same size.

Hench: In our experiments on powders of the same size in the same experiments with macrophages, we did not get macrophage death with positively charged Bioglass surfaces as we observed with negatively charged quartz surfaces (Wilson & Pigott 1981). This suggests that negative surface chemistry is related to high concentrations of negative charges.

Richards: The particular asbestos that we used has a positive charge. All other forms of asbestos have negative charges.

Wilson: There is no reason to suppose that the mechanism is going to be the same for all surfaces. We did some work with polymers and found negative charges were very toxic when the charged material was phagocytosed.

Williams: There are three or four ways of putting negative charge on organic surfaces. For example, many polysaccharides carry sulphate negative charge. Sulphate seems relatively innocuous compared with carboxylate. It looks as if biology goes around deliberately sulphating certain external surfaces. That could be to do with cation interactions. Sulphate doesn't interact strongly with cations whereas carboxylate interacts much more strongly.

Volcani: Receptors on the cell surface may be important and these are what

one would look for.

Williams: That means we need to discuss genetic manipulation as well.

Werner: New molecular genetical methods such as site-directed mutagenesis (Tn 5), identification of small fractions of mRNA by monoclonal antibodies after *in vitro* translation, and cDNA cloning, are now available for identifying the genes responsible for structural and functional proteins connected to silica metabolism, on the trace element side as well as on the macrocomponent side of mineralization. The main point is to select the right process. The most obvious one to start with is the silica uptake system. Cell types known to have an active uptake system for silica should be compared with cells which have none. The molecular genetical methods I mentioned could be applied to this carrier protein for active silica uptake. That approach can be made now.

Volcani: We now have a feasible direct approach to studying the molecular role that silicon plays in gene expression, as we have previously shown to be the case. We don't know which genes are involved and we don't know whether silicon has a direct effect or an indirect effect. We are studying this in great detail to try and isolate and characterize these genes.

Williams: A major problem arises from the conflicting statements made about the relative interactions or roles of aluminium and silicon.

Espie: Most of the people who have considered the interactions between aluminium and silicon have started from the basis that aluminium is toxic. Could you explain a bit more about your recent work on the essentiality of aluminium for animals, Dr Carlisle?

Carlisle: Aluminium, like silicon, seems to be required in very small amounts as a trace element. This doesn't really conflict with the other statements. For example, selenium is an important trace element but the difference between what is required and what is toxic is minute. There is a possibility that aluminium may be toxic in some instances because it interferes with the functions of silicon. This doesn't mean that aluminium can't still have a function at a lower concentration. We have talked a lot here about silicon toxicity but I believe that small amounts are essential. I think it is somewhat the same with aluminium.

We find that in certain tissues the constant ratio of aluminium to silicon is striking. This has been shown in five or six species by hundreds of analyses over 12–15 years. In these tissues, aluminium and silicon are at relatively high levels and have the same constant ratio, in comparison to other tissues in the same animals, even when these tissues are taken from animals on a very low aluminium and silicon diet. Furthermore, when I first worked to produce silicon deficiency in chicks aluminium was present in the diet in very low amounts and at one point when aluminium oxide was added the growth of the chicks was stimulated.

Sullivan: I agree with the general idea that Dr Carlisle is putting forward. There is a lot of supporting evidence from people studying toxic inorganic compounds in phytoplankton. There are levels that are essential for normal

function in cells and in excess of that level those same elements become toxic. F. Morel at MIT has shown that the biological effect is highly dependent not on the total amount of these ions in the aqueous solutions surrounding the cell but on their ion activity. When we talk about these various effects of aluminium we need to know what the speciation is, what the ion activity is outside and inside the cell, or whether these compounds are excluded from the cell cytosol by being chelated or sequestered in the membranes. Just knowing the total amount of mineral associated with the cell could mislead us until we know more about the chemistry.

Williams: All elements are toxic at a certain level and that level depends on the organism. The general dose-effect relationships are shown in every text-book on trace elements. But is aluminium poisonous at all levels or is it essential at very low levels?

Volcani: There is a difference between *in vitro* experiments and *in situ* or *in vivo* experiments. In some cases one can learn a great deal from *in vitro* experiments but these conditions are very different from those in the intact animal. The concentrations required *in vitro* are utterly different from those required *in vivo*. Moreover the capacity of the cell to regulate differentially *in situ* is quite different for any one of these elements from what it is *in vitro*. Professor Birchall's experiments show results that are quite different from what probably happens *in vivo*.

Espie: Yes, but there must be a simple chemical reaction comparable with *in vitro* high concentration experiments and we should be able to predict possible chemistry *in vivo* from known chemistry, determined in the test tube.

Volcani: We don't really know where this particular element is in intact animals so the *in vivo* environment is not known, whereas its *in vitro* environment is controlled.

Birchall: Nobody has yet found any bio-organic chemistry of silicon. Nobody has identified any strong binding of silicon to organic molecules under phy-siological conditions although people have looked for a long time. Now we know from dialysis studies that low levels of aluminium are toxic and we know that aluminium and silicon have a peculiar affinity in solution chemistry. We need to apply Occam's razor, throw out the hypothesis that we cannot demons-trate and bring in a new hypothesis that makes chemical sense and is ex-perimentally tractable. We have drawn attention to the interactions of silicic acid with metal ions (especially aluminium) for at least this provides the beginnings of an explanation of several observations. Of course, much more work is needed. In dialysis, it is possible that aluminium in the presence of silicic acid is not dialysable and does not enter the body. In which case we should look not at total aluminium concentration alone but at the ratio of Al and Si. The same may be true regarding the toxicity of aluminium to fish. It may be that the balance of these two elements is important so that when Dr Carlisle takes silicon out, she unmasks the toxicity of aluminium, specifically in connec-tive tissue synthesis and osteogenesis.

Carlisle: I think there is a great deal to that work. Our studies show that there is a very close homeostatic control of aluminium and silicon in the body. For elements which are only environmental contaminants there is no homeostatic control.

Richards: Are we saying that there has to be a direct chemical bond between biological components and silicon? Are we saying that absorption of materials is not important?

Williams: No. Ammonium ions are picked up by specific reagents which don't bind covalently except via hydrogen bonding.

Richards: So it may be important that something like quartz or amorphous silica binds agents in a very loose manner and then releases them again.

Hench: Large international efforts are currently under way to develop silicon nitride and silicon carbide materials for heat engines and gas turbines. These efforts could result in the release of large quantities of airborne particles with partially oxidized silicon nitride and silicon carbide surfaces. No definitive toxicity studies seem to have been done to guard against this potential danger. In our preliminary studies we found that the potential toxicity of such particles in peritoneal injections was even greater than that of quartz particles. More work on this subject is needed.

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