tween electron density and scale height, it also contains the mean pressure to the third power in the numerator, and it is not at all sure that this is constant under the conditions described.

One possible mechanism for the volcanic dust–atmosphere interaction is the absorption of solar radiation by an aerosol layer and the consequent heating of the immediate atmosphere. The lowered air density in this region may then create an increase in the atmospheric density gradient at higher altitudes.

There seem to be two regions where the required changes could have occurred. The first is around 20 km, where the largest concentration of aerosols, or primary dust cloud, was reported. However, this is a considerable distance below the mesopause, and radiosonde measurements (*16*) did not indicate the temperature differences thought necessary to produce the required scale height changes. A secondary layer in the mesosphere (*12*) is closer to the meteor ablation region than the primary stratospheric layer and could conceivably exert a greater influence on this region. Although temperature measurements at these heights are sparse, it has been observed (*17*) that in 2½ hours these altitudes can undergo a temperature change of 60°C. The presence of aerosols will increase the absorption cross section of the atmosphere to solar radiation. Reradiation of the absorbed energy will produce a neighboring temperature increase. For a given solar energy input per unit area, the lower density in the mesosphere means that a larger temperature increase will result, and the appropriate density changes follow.

Although there now seems to be little evidence for the existence of a large-scale aerosol concentration in the mesosphere, it is interesting to note that the reappearance of the excess meteor rates every alternate year correlates with a biennial oscillation of the equatorial stratospheric winds (*14*), which could conceivably cause injection of fine particles into the mesosphere on alternate years.

Another mechanism producing atmospheric heating with the correct seasonal phase is the summer penetration of solar ultraviolet radiation to a lower level at the present mid-latitude observational sites (*18*). The required heating may have been produced by volcanic augmentation of the SO_2 or SO_3 in the thermosphere (*19*), plus an increase in solar ultraviolet due to the drop in ozone concentration observed at the same time as the presence of the aerosols (*20*). Even if the presence of a mesospheric dust layer is not confirmed, heating of the lower atmospheric layers by these two effects might well cause an upward progression of the expansion to meteor heights.

Our aim in presenting this report was to show that there is a strong correlation between the increased meteor rates and volcanic dust at high altitudes. We hope that atmospheric dynamicists might be sufficiently interested by this report to investigate in greater detail the actual mechanisms involved in this interaction.

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Synthesis of Cell Wall Microfibrils in vitro by a "Soluble" Chitin Synthetase from Mucor rouxii

Abstract. *A "soluble" form of chitin synthetase was separated from a membrane-rich fraction by exposure to the enzyme substrate (uridine diphosphate* N-*acetyl*-D-*glucosamine) and activator (*N-*acetyl*-D-*glucosamine). The solubilized enzyme catalyzed the synthesis of chitin microfibrils similar, if not identical, to those formed in vivo by the fungus. Cell wall microfibrils were thus abundantly formed in the absence of a living cell or its membranes.*

Microfibrils are the skeletal components of the cell walls of the vast majority of fungi, algae, and higher plants (*1*). Cellulose and chitin microfibrils occur most abundantly and have been studied most extensively. Yet, the mechanism of elaboration of cell wall microfibrils is largely unknown.

It has been repeatedly demonstrated, by using radioisotopes, that minute amounts of insoluble products with the chemical properties of chitin (*2–4*) or cellulose (*5, 6*) can be synthesized in vitro from the appropriate nucleoside diphosphate sugar. Hitherto, however, there had been no conclusive (that is, physical) evidence that microfibrils were formed in such experiments. Moreover, claims for the biosynthesis of cellulose in cell-free extracts of plants could not be confirmed by x-ray diffraction analysis (*7*).

The subcellular site of cell wall microfibril formation is also unresolved. It is considered unlikely that microfibrils are elaborated internally and then secreted in prefabricated form (*8*) except in some unusual systems (*9*). It is generally believed that microfibrils are assembled at the cell surface (*8*) and that the plasmalemma is actively engaged in their synthesis (*10*). Studies with cell-free extracts have shown that cell wall polysaccharide synthetases are associated with various membrane fractions (*3, 4, 6, 7*), but localization of the synthesizing enzymes in the wall itself has also been reported (*4, 11*). Invariably, little or no activity is found in the soluble cytoplasm. Repeated efforts to solubilize chitin synthetase failed, except perhaps that of Glaser and Brown (*2*), who not only discovered chitin synthetase but were also successful in solubilizing

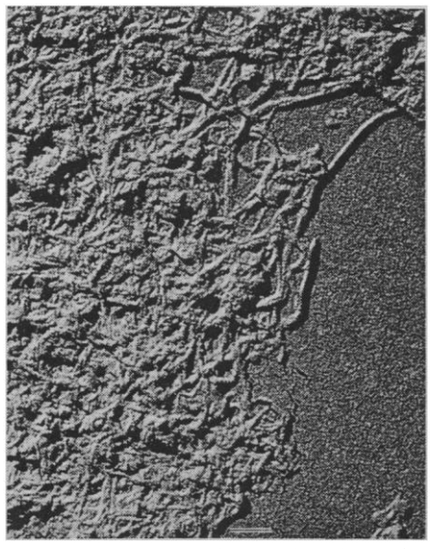

Fig. 1. Electron micrograph of chitin microfibrils synthesized in vitro. The sample was washed with cold 1*N* NaOH to remove enzyme, mounted on a grid, and shadow cast with Pd at 19°. Scale bar, 200 nm.

it with butanol. Whether the solubilized enzyme formed microfibrils was not ascertained.

We now present evidence for the synthesis of chitin microfibrils in vitro by a "soluble" (*12*) enzyme prepared in an unusual way. Separation of the enzyme from membrane-rich fractions was achieved while testing a working hypothesis on wall fibrillogenesis, namely, that a polysaccharide synthetase would be released from a postulated initial association with the plasmalemma (or other membranes) the moment it started elaborating a microfibril, that is, on exposure to its substrate and activator (*13*).

This work was done with the yeast form of *Mucor rouxii*, whose chitin synthetase has properties similar to those of the mycelial enzyme previously studied (*4*, *14*)—one important difference being the greater stability of the yeast enzyme (*15*).

Yeast cells of *M. rouxii* (*16*) were grown under an atmosphere of 30 percent CO_2 and 70 percent N_2 for 12 hours, harvested by filtration, and broken in a Braun model MSK homogenizer (*14*, *15*). The homogenate was centrifuged at 2,000*g* for 5 minutes to remove cell walls and then at 35,000*g* for 15 minutes to separate a mixed membrane fraction (MMF).

The MMF contains most of the chitin synthetase activity in the cell-free extract (*15*). The enzyme is present largely in an inactive state and can be activated by proteolysis (*15*), as demonstrated earlier for the chitin synthetase of *Saccharomyces cerevisiae* (*17*).

Solubilization of chitin synthetase occurred when the MMF was incubated with substrate (uridine diphosphate *N*-acetyl-D-glucosamine) and activator (*N*-acetyl-D-glucosamine) at 0°C for 30 to 60 minutes (*18*). After centrifugation at 81,000*g* for 30 minutes, about 15 to 20 percent of the total chitin synthetase activity (but only 2 to 3 percent of the total protein) remained buoyant. (In similar mixtures incubated without substrate or activator, only 3 percent of the total enzyme activity was solubilized.) When this soluble supernatant was brought to room temperature, turbidity appeared in about 20 minutes, followed by the precipitation of a fibrous material. Using radioactive substrate in the incubation mixtures (*18*), we found about 30 percent polymerization after incubation for 1 hour (chitin yield per incubation mixture = 0.7 mg). After partial acid hydrolysis and *N*-acetylation of the product, paper chromatography showed a typical series of chitin oligosaccharides (*4*).

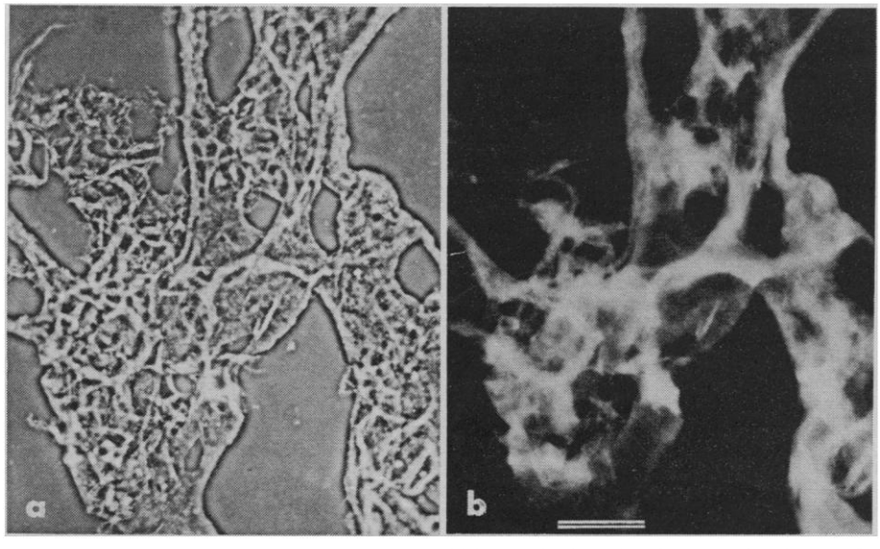

Fig. 2. Light micrograph of a specimen of chitin synthesized in vitro (a) by Anoptral phase contrast and (b) under ultraviolet light (stained with Calcofluor white M2R). Scale bar, 50 μm.

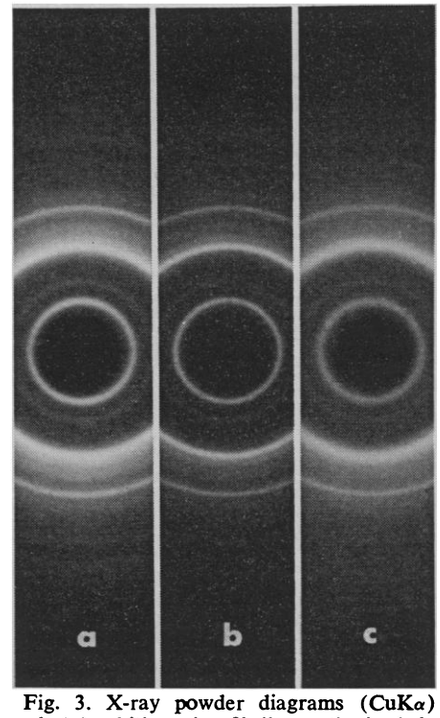

Fig. 3. X-ray powder diagrams (CuKα) of (a) chitin microfibrils synthesized in vitro (sample washed with cold 1*N* NaOH), (b) a highly purified sample of crab chitin, and (c) chitin from yeast cell walls of *Mucor rouxii* (*27*).

Electron microscopy revealed that the product thus synthesized consisted of networks of highly regular, long microfibrils about 20 nm in diameter (Fig. 1). Some of the networks showed a striking resemblance to pieces of incipient cell walls formed in vivo (*19*). Light microscopy showed mostly pellicles of many different shapes (Fig. 2a). Also observed were thick fibers and numerous isolated particles (1 to 2 μm). The synthesized material, regardless of shape, stained intensely with Calcofluor white M2R (Fig. 2b), a fluorescent stain for fibrillar wall polysaccharides (*20*). X-ray diffraction analyses proved conclusively that the biosynthesized product was α-chitin of a crystallinity comparable to that of highly purified chitin (*Cancer magister*) (Fig. 3). The x-ray reflections from chitin synthesized in vitro were slightly sharper than

those from chitin isolated from yeast cell walls of *M. rouxii*.

To our knowledge, this is the first report on the formation of cell wall microfibrils in vitro. Our findings (i) demonstrate that the synthesis of chitin chains and their assembly into microfibrils can be attained with a soluble (*12*) enzyme preparation free of membranes (*21*); (ii) prove, unequivocally, that the Leloir (*22*) pathway of polysaccharide synthesis (glycosyl transfer from a nucleoside diphosphate sugar) operates in the formation of cell wall microfibrils; (iii) show that cell wall microfibrils can be formed in the absence of a living cell or its membranes (*23*); (iv) support the belief that the formation of cell wall microfibrils (polymer synthesis and fibril assembly) in the living cell takes place in the wall itself (*24*); (v) suggest that other wall components, such as matrix materials, are not indispensable for microfibril elaboration (*25*); and (vi) move us a step closer to the goal of understanding, and eventually duplicating in vitro, the assembly of a whole microfibrillar cell wall (*26*).

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3 June 1974

Neuroblastoma: Drug-Induced Differentiation Increases Proportion of Cytoplasmic RNA That Contains Polyadenylic Acid

Abstract. *The production of cytoplasmic RNA that contains polyadenylic acid is increased, relative to total cytoplasmic RNA, in a neuroblastoma clone, NBE$^-$(A), after induction of differentiation by 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone, an inhibitor of adenosine 3′,5′-monophosphate phosphodiesterase. The amount of RNA that contains polyadenylic acid in cytoplasm may be greater in such differentiated neuroblastoma cells than in proliferating control cells.*

The presence of polyadenylic acid [poly(A)] sequence at the 3′ terminus of messenger RNA derived from a variety of sources has been reported (*1*). It is in the cytoplasm where RNA functions as a template in protein synthesis. Therefore we have examined the rate of appearance and amount of cytoplasmic RNA that contains poly(A) in a neuroblastoma cell line cultured in two widely differing states of genetic expression. It is possible to selectively separate the RNA's that contain poly(A) by utilizing their ability to bind to cellulose at high ionic strength (*2*).

The neuroblastoma clone NBE$^-$(A) which has choline acetyltransferase activity, but no tyrosine hydroxylase activity (*3*), was cultured and maintained as described (*4*). These cells have a doubling time of about 18 hours, and they produce tumors when injected subcutaneously into male A/J mice. Cells were plated in Falcon plastic flasks (75 cm^2); 4-(3-butoxy-4-methoxybenzyl) - 2 - imidazolidinone (RO20-1724, 200 μg/ml), a rather specific inhibitor of cyclic AMP phosphodiesterase (*5*), was added 24 hours after the plating. This drug results in an elevation of intracellular adenosine 3′,5′-monophosphate (cyclic AMP) and in increases of the enzyme activities of several enzymes, including some specifically associated with neural function (*4*). In addition, RO20-1724 induces many morphological indices of differ-