(*7*). In females, the picture is complicated by a further increase in hemoglobin concentration and specific gravity accompanying the maturity of the ovarian eggs. In the same way, the glycogen concentration of the liver is higher per gram of tissue in larger animals than in small (*8*).

All in all these results show that as auxetic growth continues, concomitant physiological changes occur which probably affect the metabolism of the animal. To clarify this conclusion, the effects of environmental changes in temperature, dehydration, and diet, and possible changes in the serum proteins, are being investigated in addition to the studies mentioned above.

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27 January 1961

Synthesis of Bacterial Cellulose from Labeled Precursor

Abstract. The isolation and purification of an immediate precursor of bacterial cellulose was confirmed with glucose randomly labeled with carbon-14. The glucose appears to be bound within the cell to a lipid, is carried across the bacterial cell wall, and is incorporated enzymatically into cellulose extracellularly.

The isolation and purification of a compound containing glucose bound to a lipid, from ethanol extracts of active suspensions of *Acetobacter xylinum*, has been described recently (*1*). This compound appears to be the precursor of bacterial cellulose in that it will form typical microfibrils in aqueous solutions containing an extracellular enzyme. Preparations of the compound were shown to be homogeneous by two-dimensional chromatography in an acidic and a basic solvent, as tested by four detector systems, including autoradiography. We report here confirmation of the previous work from the results of a study of the transfer of glucose randomly labeled with C^{14} from the above compound to cellulose, under conditions which simulate in vivo formation of microfibrils. These results afford additional insight into the mechanism of biosynthesis of cellulose by this bacterium.

Labeled precursor was prepared as follows: Cellulose-free, washed cells from 100 ml of a suspension of *A. xylinum*, prepared as described previously (*2*), were incubated for 4 or 5 min (depending on the activity of the individual cell suspension) at 35°C in a 2 percent glucose solution, 0.01*M* in phosphate buffer, *p*H 6.0. Sufficient glucose in this solution was randomly labeled with C^{14} to give radioactivity of 2.5 μc/ml of initial solution. The isolation of the precursor from ethanol extracts of these suspensions, in the fourth fraction off a magnesium trisilicate-Celite column, M4, was carried out as reported earlier (*1*). For autoradiography of the labeled preparations, paper chromatograms (acid-washed Whatman No. 1 paper 7 by 7 in.) were developed two-dimensionally by the descending technique in *n*-butanol, acetic acid, and water (4:1:1) and *n*-butanol, pyridine, and water (10:3:3). The chromatograms were then placed in contact with medical x-ray film, which was developed after 4 days of exposure. Quantitative estimates of the fraction of glucose incorporated into the precursor at any instant were not undertaken because the fraction is very small and the compound is sensitive to traces of water on the paper. For instance, drying the preparation on initially slightly damp chromatographic paper will completely destroy it.

The fraction containing the precursor of bacterial cellulose, M4, exhibited only one spot on the autoradiograms in the same position as the compound detected previously (*1*) by KIO_4-starch reagent (*3*). At low concentrations round compact spots were observed, but at higher concentrations severe streaking of the chromatogram was always present. This streaking is attributed both to a slow breakdown of the compound in the water phase and to a slow rate of attainment of equilibrium. No traces of the predominant compounds in the suspension medium, glucose, and the gluconates were detectable in the autoradiograms of the M4 fraction.

The incorporation of a component of M4 fraction into bacterial cellulose, under certain conditions, was demonstrated as follows: (i) 1 ml of M4 incubated with 1 ml of water for 30 min at 35°C; (ii) 1 ml of M4 incubated as above with 1 ml of an ultrafiltered supernatant fraction of an active culture which contains an enzyme catalyzing cellulose formation (*4*); (iii) 1 ml of ultrafiltered supernatant fraction incubated alone for 30 min at 35°C. After incubation, each sample was diluted to 10 ml with redistilled absolute ethanol and centrifuged at 15,000 *g* for 15 min. The pellet, if any, was re-extracted with 3 ml of chloroform to remove lipids and recentrifuged.

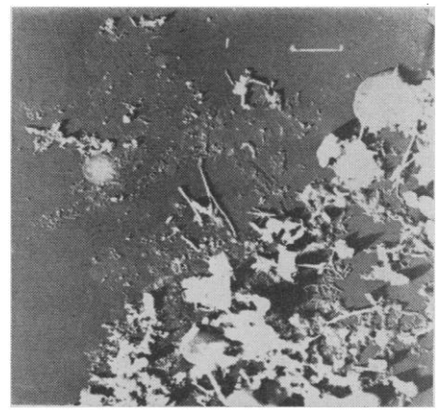

Fig. 1. Typical chloroform-ethanol insoluble, alkali-resistant, amorphous material formed by M4 fraction in the presence of only water.

Ethanol-chloroform insoluble material was formed principally in (ii), very little in (i), and none in (iii). The pellets from (i) and (ii) were digested with hot 4 percent NaOH to remove noncellulosic polymers (*4*), washed free of NaOH, dispersed in water,

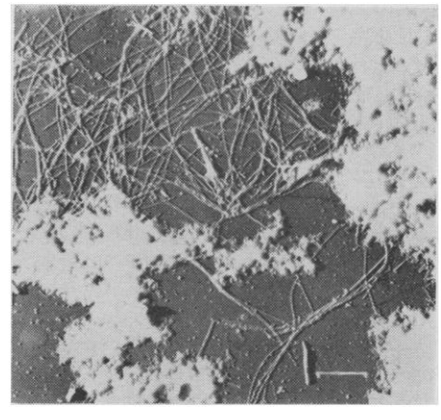

Fig. 2. Typical chloroform-ethanol insoluble, alkali-resistant cellulose microfibrils formed by the M4 fraction in the presence of an enzyme in the ultrafiltered supernatant fraction of an active culture of *A. xylinum*. Note the presence of substantial amorphous material also.

dialyzed, mounted, and shadowed for examination in the electron microscope. Most of the material from (i) dissolved in hot NaOH and showed only traces of amorphous substance (Fig. 1). Conversely, material from (ii) was fibrillar, resembling normal bacterial cellulose microfibrils (Fig. 2), and gave a positive anthrone reaction (*5*). Radioactivity was detected when the microfibrillar material was spread on a planchet under a counter and when it was exposed as a thin film to an x-ray emulsion. The combined ethanol and chloroform extract from (i) was radioactive, but no radioactivity was detectable in the combined extracts from (ii).

These results confirm the previous conclusion that the compound in the M4 fraction is an immediate precursor of bacterial cellulose. They also confirm the conclusion that a catalyst, which is probably an enzyme, is required for the formation of the cellulose microfibrils from the precursor, and that this enzyme is present in the extracellular medium of *A. xylinum*. This conclusion has been substantiated recently for *A. acetigenum* also (*6*). In the presence of the enzyme, the glucose is transferred mainly from soluble precursor into insoluble cellulose microfibrils by tip growth (*7*). No evidence for the necessity of a primer was observed, but these experiments do not exclude this possibility. These results thus support the postulation of a diffusible intermediate by Schramm *et al.* (*8*).

Presumably the precursor, containing lipid-bound glucose, is formed within the cells from glucose taken up during incubation. The bound activated glucose is then transported across the cell wall, perhaps aided by the presence of a substantial lipid moiety. After the extracellular enzymatic transfer of the glucose portion of the molecule to the growing microfibril, the lipid fraction is probably recycled since it does not accumulate in the medium (*9*).

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Nondestructive Method for Estimating Chlorophyll Content of Leaves

Abstract. A quantitative relationship is shown to exist between the chlorophyll content of soybean and Valencia orange leaves and their percentage reflectance of light of wavelength 625 mμ as measured by a colorimeter with reflectance attachment.

Incident to an investigation of the response of plants to iron supply, a method was needed whereby successive daily estimates of chlorophyll content could be made on the same leaf. Standard methods were not suitable because they require destruction of the leaf. Since chlorotic leaves become greener to the eye as the chlorophyll content increases, it seemed likely that reflectance of certain wavelengths from a leaf surface might be sufficiently well correlated with the chlorophyll content to serve as an estimate of that content. Reflectance measurements of leaves have been reported (*1*), and a general relationship has been recognized between the percentage of incident light reflected and the chlorophyll and carotenoid pigment concentrations (*2*). Apparently no attempt has been made to utilize this relationship to obtain quantitative indications of changes occurring in the chlorophyll content of leaves.

The basic instrument used was a colorimeter (Bausch and Lomb Spectronic 20) equipped with a reflectance attachment. It was soon established that the least difference in reflectance from the under surfaces of green and chlorotic soybean leaves occurred at wavelengths of about 665 and 465 mμ, while the greatest difference occurred at a wavelength of about 570 mμ. These wavelengths occur in the regions of maximum and minimum absorption of chlorophyll and agree with the results cited by Gabrielsen (*2*). Since the greatest difference in reflectance occurred at 570 mμ, this wavelength seemed to be the one to employ in developing a relationship between chlorophyll content and reflectance. It was found, however, that a still greater spread in reflectance from green and chlorotic leaves could be obtained by using a wavelength of 625 mμ and placing a filter with peak transmission at that wavelength between the leaf and the white magnesium carbonate reflecting surface.

To establish quantitative relationships between reflectance percentage and chlorophyll content, reflectance was measured for several hundred soybean leaves exhibiting different degrees of chlorosis induced by iron deficiency. These leaves were then sorted into groups. All leaves with reflectance readings between 33 and 37 percent (average 35) were placed in one group, those between 38 and 42 percent in the next group, and so on. Replicate determinations were made of the chlorophyll content of each group, by the spectrophotometric method described by the Association of Official Agricultural Chemists (*3*). The coefficient of variability of chlorophyll content within a group was found to be 8 percent. The results of these determinations (Fig. 1) show a high inverse relationship between the logarithm of the reflectance percentage and the chlorophyll content of the leaves within the range of concentrations studied.

To test the method on a different type of leaf, Valencia orange leaves were used. These leaves, exhibiting different degrees of chlorosis, regardless of the cause, were collected at random from local trees. The relationship between reflectance readings and chlorophyll content was found to hold about as well for these leaves (see Fig. 1) as for soybean leaves, except at the higher levels where the straight-line relationship no longer held.

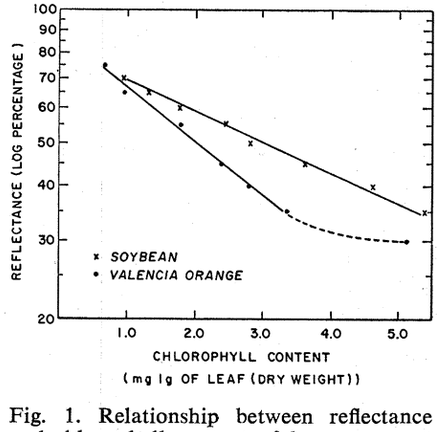

Fig. 1. Relationship between reflectance and chlorophyll content of leaves.