Table 1. Effect of treatment with IAA, in the presence of nitrogen or at low temperature in basal media of distilled water or calcium chloride, on deformation of wheat coleoptile sections. Each result is the mean of the deformations of ten sections per treatment. Differences between means were taken as significant at the 5-percent level.

Expt. No.	Basal medium	Mean total angle of deformation (deg)						
		Before treatment (25°C)	After treatment in					
			Basal medium alone	Basal medium + IAA	Basal medium + N_2	Basal medium + N_2 + IAA	Basal medium at 3°C	Basal medium at 3°C + IAA
1	Distilled water	25	27	40	11	23		
2	Distilled water	23	25	38			18	26
3	0.02*M* $CaCl_2$	15	12	16			6	14
4	0.02*M* $CaCl_2$	32	12	17	8	16		

curred only in water or water plus auxin. Deformation data from four representative experiments are shown in Table 1. Treatment with calcium chloride solution caused a significant stiffening of the sections compared with the initial values. In both basal media deformation of auxin-treated coleoptiles was significantly greater than that of sections treated in the absence of auxin. The deformation of coleoptiles treated in nitrogen or at 3°C in a basal medium of calcium chloride solution was significantly less than the deformation of coleoptiles treated in the basal medium alone. Nitrogen also caused a significant stiffening of sections treated in a basal medium of distilled water. The deformation of coleoptiles treated in nitrogen or at 3°C in the presence of auxin was significantly greater than the deformation of such tissue treated in the absence of auxin and was not significantly different from that of coleoptiles treated in basal media alone.

The effect of auxin in reducing wall stiffening in nitrogen and at low temperature suggests an action of auxin which is independent of these conditions, which reduce metabolic activity. The increased wall plasticity and cell expansion which result from auxin action are, however, metabolically dependent. Changes in cell-wall plasticity under different conditions may be interpreted in terms of pectin methylation and demethylation reactions, as is illustrated in Fig .1.

The activity of PME is insensitive to a large number of inhibitors (*4*), and pectin demethylation may occur independently of metabolic conditions. Because of a requirement for adenosine triphosphate, methylation is sensitive to metabolic conditions. A greater sensitivity of methylation compared with demethylation would result in a decrease in the methyl content of pectin in the presence of nitrogen or at low temperature. The increased number of carboxyl groups available for divalent cation binding would result in the observed wall stiffening under such conditions.

The effect of auxin in reducing wall stiffening at low temperature or in nitrogen may be due to an action of auxin which reduces the rate of demethylation and thus conserves the methyl content of pectin. The metabolic insensitivity of the action of auxin which conserves wall plasticity is consistent with Glasziou's hypothesis of an auxin-mediated adsorption reaction which reduces pectin demethylation by reducing PME activity.

It is suggested that the metabolic sensitivity of auxin-induced wall plasticization and cell expansion reflects the sensitivity of the methyl incorporation into pectin and that an auxin action which conserves wall plasticity is independent of metabolic conditions (*5*).

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5. The work described in this paper was carried out during the tenure of a university studentship in plant physiology (D. A.) and senior postgraduate studentship of the Commonwealth Scientific and Industrial Research Organization (H.A.).

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Separation of Tobacco Leaf Proteins by Centrifugation across a Density Boundary

Studies by Wildman and coworkers (*1*) have shown that two major protein fractions are present in green tobacco leaves. The two fractions, I and II, are characterized by sedimentation constants of about 19 and 4 Svedberg units, respectively. Attempts to isolate the fractions have not been wholly successful. A partial resolution can be obtained by salt precipitation (*2*), and fairly pure fraction I has been obtained by repeated ultracentrifugation, but in very low yield and in a highly aggregated form (*3*). This report (*4*) describes a simple procedure for separating the two fractions by ultracentrifugation across a density boundary (*5*).

In principle, a stable boundary is formed by layering a tobacco extract over a buffer-salt solution of greater density. In view of the difference in the sedimentation constants of the two fractions, it is then possible to choose centrifugation conditions such that the faster moving fraction I is completely sedimented, whereas the more slowly sedimenting fraction II moves only a short distance below the initial boundary. After centrifugation the upper portion of the tube should contain only fraction II, and fraction I should be concentrated in the pellet. The middle portion—that is, the region from the initial boundary to that just above the pellet—should contain some fraction II and probably some fraction I because of convection. The procedure and the results obtained in an actual separation run are described below.

The laminar portion of a sample of mature Burley tobacco leaves was lyophilized to a moisture content of 7.66 percent and ground in a Wiley mill. An extract was prepared by grinding 0.3 g of the tissue with 10 ml of 0.2*M* potassium maleate buffer, *p*H 7.5, in a Ten Broeck homogenizer. The buffer contained 0.2 g of $Na_2S_2O_4$ per liter as a color inhibitor, and the extract was maintained at about 7°C throughout the grinding and subsequent manipulations. Seven such extracts were pooled, and cell debris and other insoluble material were removed by centrifugation in a Spinco C rotor at 42,040 rev/min (av. 130,000 *g*) for 1 hour. A thin, oily, green layer at the surface of the liquid was removed by slicing off the upper portion of the Lusteroid tubes with a Spinco tube slicer; the faintly hazy supernatant fluid was then filtered through a 0.5-μ Millipore filter (*6*) to remove the suspended particulate matter. This clarified extract was clear and light brown in color and contained 0.85 mg of N per milliliter, which constituted approximately 60 percent of the total nitrogen of the original sample. A portion of the filtrate was mechanically dialyzed for 6 hours against three portions (two changes) of extraction buffer (without $Na_2S_2O_4$) and then analyzed for total nitrogen and examined in the ultracentrifuge.

For the separation run, seven tubes were prepared. Four milliliters of the clarified extract were added to each tube. A syringe, filled with extraction buffer, which was also 0.2*M* in K_2SO_4, was carefully inserted so that the tip of the needle was close to the bottom of the

tube, and 5 ml of this denser solution was then slowly injected below the extract. A sharp and relatively stable boundary was thus formed between the colored extract and the colorless buffer-salt solution. Centrifugation was carried out in a Spinco C rotor at 42,040 rev/min for 6 hours at 7°C. The color boundary, which appears to be associated with the movement of fraction II, is blurred somewhat during the centrifugation. By means of the tube slicer, five of the tubes were sampled as follows, and the corresponding fractions were pooled: top 4 ml, middle 4 ml, and lower 1 ml plus pellet. An additional 1 ml of buffer was used to rinse the lower portion of each tube, so that the total volume of the pellet solution was 10 ml. The solutions were dialyzed against maleate buffer as indicated above, and the pellet solution was subsequently centrifuged at 25,000 *g* for 30 minutes at 7°C to remove a small amount of insoluble green material. The two remaining tubes from the separation run were used to examine the composition of the pellet itself. The supernatant fluid was simply decanted, and the pellets were dissolved in a total of 8 ml of maleate buffer. The resulting solution was then dialyzed, and the insoluble green matter was removed as described above. All solutions were analyzed for total nitrogen and examined in the ultracentrifuge.

Table 1. Ultracentrifugal and analytical data for an extract of green Burley tobacco and for various fractions obtained after separation ultracentrifugation. To facilitate comparison of the data, peak areas and nitrogen values are all expressed in terms of 4 ml of the clarified extract.

Solution	Determination		
	N (mg)	Fr. I area (cm^2)	Fr. II area (cm^2)
Clarified extract, before separation run	2.06	21.6	54.0
Fractions, after separation run:			
Top 4 ml	0.94		46.0
Middle 4 ml	0.47	4.4	8.4
Lower 1 ml plus pellet	0.60	10.6	2.6
Pellet only, after separation run	0.21	3.2	0.8

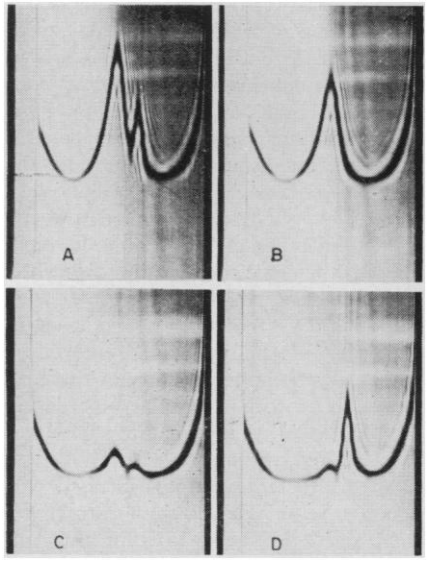

Fig. 1. Schlieren patterns of tobacco extracts to illustrate separation of protein fractions by centrifugation across a density boundary. Sedimentation is from left to right; the faster-moving component is fraction I. *A*, clarified extract before separation run, showing both fractions I and II; *B*, top 4 ml from separation run, pure fraction II; *C*, middle 4 ml from separation run, showing traces of both components; *D*, lower 1 ml of tube plus pellet (solution diluted 1:1 with maleate buffer), primarily fraction I. Patterns photographed at 35° bar angle and 8 minutes after the rotor had reached speed (50, 740 rev/min). Runs were made in a synthetic boundary cell (see *5*) at 25° ± 0.4°C.

Sedimentation patterns obtained for the clarified extract and for solutions from the separation run are shown in Fig. 1. Analytical data, including that for the pellet only, are summarized in Table 1. Peak areas, which are proportional to the concentration of the sedimenting components, and sedimentation constants were evaluated from enlarged tracings of the solution and base-line patterns. The areas were corrected for the dilution effect, but Johnston-Ogston corrections (*7*) were not made. The average sedimentation constants determined for fractions I and II were 17 and 3 Svedberg units, respectively.

Analysis of the data shows that the over-all recovery of nitrogen from the clarified extract is 97 percent. The recoveries of fractions I and II, calculated from the peak area measurements, are 70 and 105 percent, respectively. The lower apparent recovery of fraction I is believed to be associated primarily with the uncertainty entailed in estimating the precise volume of the "lower 1 ml" when the tube-slicer technique is utilized. It is also apparent that a major portion of fraction I is suspended in the solution which bathes the pellet and that the fraction I in this solution is not entirely free of fraction II. These factors may be accounted for in terms of sedimentation theory for angle-tube centrifugation and could probably be eliminated or minimized appreciably by the use of a swinging bucket rotor to provide purely horizontal centrifugation.

This relatively simple procedure, with variations, has been carried out many times. The yields of both fractions have been uniformly high, and the preparations of fraction I have been essentially colorless and have been characterized by little or no aggregation (see Fig. 1 *D*). While the present application has been confined to the proteins of green tobacco, the method should be generally applicable to the separation of other mixtures containing macromolecular constituents whose sedimentation constants vary appreciably.

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5. The separation procedure employed in this study is somewhat similar to that suggested by M. K. Brakke [*J. Am. Chem. Soc.* **73**, 1847 (1951)], in which a solution is layered over a solvent having a density gradient. The synthetic boundary cell, developed by E. G. Pickels *et al.* [*Proc. Natl. Acad. Sci. U.S.* **38**, 943 (1952)], is based on the same principle except that a solvent of uniform density is layered over the solution.
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Identification of Growth-Promoting α-Hydroxy Fatty Acids Produced by Lactobacillus casei

It was reported in an earlier communication from this laboratory that practically all of the growth-promoting activity in extracts of *Lactobacillus casei* 7469 culture filtrates, as measured with *L. casei* 280-16A (a D-α-hydroxy fatty acid-dependent strain), was associated not with lactic acid but with a more lipophilic substance considered likely to be a higher homolog of lactic acid (*1*). A more rigorous fractionation of such extracts in the present investigation (*2*) has now revealed that they contain at least three growth-promoting acids, two of which appear to be identical with D-α-hydroxyisovaleric and D-α-hydroxyisocaproic acids, respectively.

Lactobacillus casei 7469 was incubated at 35°C for 24 to 96 hours, and the resulting cultures were clarified by passage through a Sharples supercentrifuge. Hydrochloric acid was added (180 meq/liter) to the essentially cell-free solutions, and the acidified media were subjected to continuous liquid-liquid extraction with isopropyl ether. The extracted material from each culture was fractionated by a 200-transfer countercurrent distri-