ENZYMATIC ACTIVITIES ASSOCIATED WITH CELL WALL PREPARATIONS FROM CORN COLEOPTILES [1, 2, 3]

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An effect of auxins on the properties of plant cell walls has been proposed as a mechanism for auxin-induced extension growth (17, 5, 30). Implicit in this point of view would be the localization of enzymes within the walls capable of synthesizing cell wall constituents or metabolically altering them. Some enzymatic activities of a cell wall preparation are reported in the communication.

Numerous cytological studies indicate that synthesis of cell wall components may occur exterior to the cell membrane. A few examples of apparent extraplasmatic cell wall growth are:

I. Glandular hairs of Labiatae (Mentha, Salvia, Thymus, etc.) may have an eightfold increase in size without a decrease of cuticular thickness, although this layer is separated from the cytoplasmic membrane and primary cell wall by a noncytoplasmic emulsion (34, 36).

II. In Isoetes and Selaginella, the cellulosic intine, and cuticular exine of the macrospore increase in surface and thickness without being in direct contact with cytoplasm or a cytoplasmic membrane (11). Similar observations have been made by other investigators on Marsilia, liverworts, and even on the pollen grains of higher plants (21).

III. The cellulosic sorophore sheath of *Dictyostelium discoideum* is formed extracellularly (29, 13).

IV. Cellulose synthesis in *Acetobacter xylinum* takes place in the external medium at a distance from the cytoplasmic membrane (25, 15, 7).

V. Dippel (9) demonstrated that the primary and tertiary cell walls appeared physically and chemically identical. The pit cavities and the inner surface of the cell are coated by a tertiary cell wall layer. This was considered by Dippel and later by others (3) as suggesting that the secondary cell wall was synthesized extraplasmatically.

VI. Uniform incorporation of C^{14}-sucrose into cellulosic and noncellulosic material throughout the thickness of the elongating outer walls of epidermal cells of Avena coleoptiles (33) demonstrates that wall synthesis is not confined to the zone proximal to the protoplast.

In addition to the cytological data suggesting extraplasmatic cell wall growth there are numerous reports of enzymatic activities associated with cell wall fractions from higher plants, as for example, acid phosphatase (22), invertase (4), ascorbic acid oxidase (35, 27, 6) and pectin methyl esterase (2, 14). Some enzymes of bacteria, fungi, protozoa, and erythrocytes, such as invertase, lactase, trehalase, phosphatase, including adenosine triphosphatase (ATP-ase[4]), choline esterase, proteolytic enzymes, dehydrogenases, diphosphopyridine nucleotidase, ascorbic acid oxidase, and pectin methyl esterase are associated with the external cell surface (37, 24, 31, 12).

Experiments suggesting that enzymes are located in cell walls are difficult to evaluate owing to the possibility of enzyme adsorption or adhesion of cytoplasmic constituents to the walls. Nonetheless the cytological studies discussed above, the in vitro enzymatic studies previously reported, as well as these presented here, and growth experiments indicating changes in the physical state of the walls as a concomitant of growth are, taken together, very convincing. We have adopted, as a working hypothesis, the premise that enzymes synthesizing cell wall constituents are, in fact, located in the cell walls. A previous publication from this laboratory reported a method for preparing cell walls free of visible contamination by other cell constituents (20).

MATERIALS & METHODS

All preparative procedures of fractions to be assayed for enzyme activity were conducted at 1 C. Corn coleoptile cell walls were prepared as previously described (20) using 80 % glycerol as the homogenizing medium and glycerol as the washing medium.

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[1] Received revised manuscript May 5, 1961.

[2] Journal Article 2701 from the Michigan Agricultural Experiment Station.

[3] We gratefully acknowledge support from the National Science Foundation.

[4] Abbreviations to be used include: ATP-ase for adenosine triphosphatase, ATP for adenosinetriphosphate, ADP for adenosinediphosphate, AMP for adenosinemonophosphate, UTP for uridinetriphosphate, UDP for uridinediphosphate, UMP for uridinemonophosphate, UDPG for uridinediphosphoglucose, G-1-P for glucose-1-phosphate, PP for pyrophosphate, Pi for inorganic phosphate, tris for tris(hydroxymethyl)aminomethane, and EDTA for ethylenediamine tetraacetate.

An essentially non-aqueous medium was chosen to minimize elution of protein from the wall fragments. Such preparations contain an average 0.6 % nitrogen as determined by micro-Kjeldahl (23).

Coleoptile homogenates were prepared by grinding the tissue with an equal weight of 0.01 M potassium phosphate, pH 7.5, or 0.01 M tris-0.001 M EDTA, pH 7.5, for 1 minute in a high speed homogenizer (Omnimixer). For soluble protein and particulate enzyme preparations, 25 g of tissue were ground in a mortar and pestle with an equal weight of buffer (as above), filtered through gauze and centrifuged at 500 × *g* for 5 minutes. The plastid fraction was collected by centrifuging for 15 minutes at 1,000 × *g*. Recentrifugation of the supernatant fluid for 30 minutes at 25,000 × *g* yielded the mitochondrial fraction. Plastid and mitochondrial fractions were then suspended in a small volume of 0.1 M tris, pH 7.5, and dialyzed against 1 to 2 liters of 0.2 % KCl for 20 hours.

Soluble protein in the supernatant fluid, after removing plastids and mitochondria, was precipitated by adding solid ammonium sulfate to the desired concentration; the precipitated protein then was collected by centrifuging at 11,000 × *g* for 15 minutes. The pellet, so obtained, was dissolved in a small volume of 0.1 M tris, pH 7.5, and dialyzed against 0.2 % KCl. This procedure is essentially that used by Neufeld et al. (26) for preparing UDPG pyrophosphorylase.

AMP, ATP, tris, and α-glycerophosphate, were from the Sigma Chemical Co.; ADP, UMP, and UTP, from the Pabst Laboratories, and EDTA from the Dow Chemical Co. Glucose oxidase (Glucostat) was purchased from Worthington Biochemical Corp., Freehold, N. J. P^{32}-labeled inorganic phosphate, obtained from the Oak Ridge National Laboratories, was used to prepare P^{32}-labeled inorganic pyrophosphate (1). The P^{32}-PP when freshly prepared had a specific activity of 1 μc/μmole (1 μc of P^{32}-PP equalled 150,000 c/min). P^{32}-PP incorporated into nucleotide was determined by charcoal absorption (8) or by elution of nucleotide after electrophoretic migration in 0.1 M acetate buffer, pH 5 at 1 C. Inorganic phosphate was determined colorimetrically (10) and glucose by use of glucose oxidase (32). Incubation of enzyme reaction mixture was at 37 C.

Results

Cell wall preparations were tested for the ability to hydrolyze inorganic pyrophosphate, α-glycerophosphate, and ATP. Pyrophosphatase activity has not previously been reported to be associated with cell walls but its presence might be expected in a tissue synthesizing UDP-sugars. Hydrolysis of α-glycerophosphate serves as an indication of nonspecific phosphatase activity while ATP-ase has previously been reported to be associated with erythrocyte membranes (28) and yeast cell walls (31). The reaction mixture contained: 7.5 mg cell wall (45 μg N); tris, pH 7.5, 15 μmoles; $MgCl_2$, 5 μmoles, and 2 μmoles of either pyrophosphate or α-glycerophosphate, or 5 μmoles of ATP. The reaction rates observed at 37 C were 6, 1, and 22 μg Pi liberated/minute/mg protein^{-1} for pyrophosphate, α-glycerophosphate, and ATP, respectively. The PP-ase activity is only 1/50 the specific activity of crude yeast extract (16) but the ATP-ase activity is ¼ that reported for muscles (19) and 25 times the activity reported for erythrocyte ghosts (28).

We compared the ATP-ase activity of the cell walls and the plastid and mitochondrial fractions. The activities of these fractions were, respectively, 10, 4, and 3 μg Pi liberated/minute/mg protein^{-1}.

A portion of the cell wall ATP-ase activity is latent (illustrated by data of fig 1). For this experiment we suspended glycerol prepared cell walls in 0.01 M tris-0.001 M EDTA and removed an aliquot for ATP-ase assay. The activity observed is labeled in the figure as CW-O. We then sedimented the cell walls by centrifuging and decanted the supernatant fluid. This procedure was repeated twice and the three supernatant fluids combined. After 24 hours at 1 C, we resuspended the walls in buffer, and tested aliquots of the wall suspension and of the combined supernatant fluids for ATP-ase activity. The activity of the walls (CW–I) was only slightly reduced

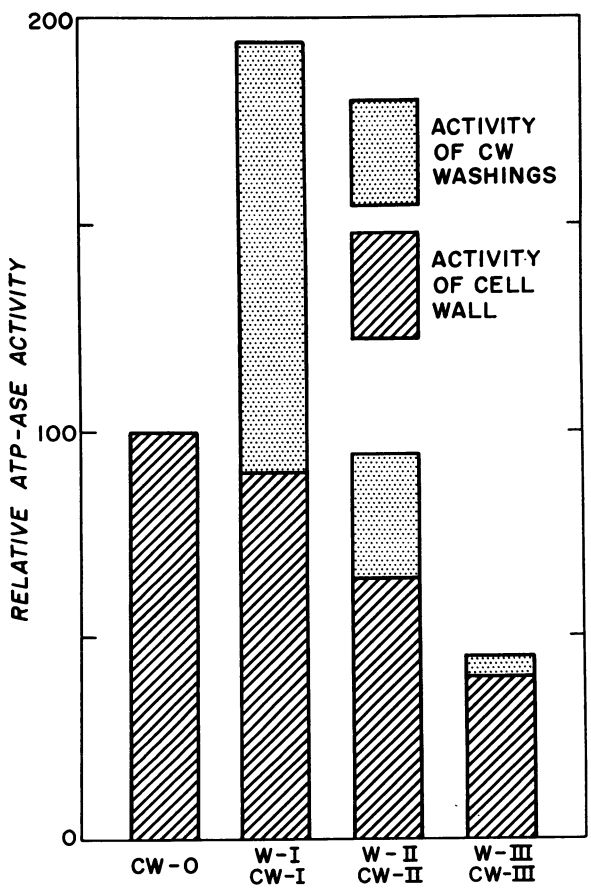

Fig. 1. The effect of washing on ATP-ase activity in cell wall preparations. Discussed in text.

by the three washes, but the combined supernatant fluids (W–I) now showed an activity approximately equal to that found in freshly suspended cell walls. There is thus two times as much ATP-ase activity after 24 hours storage at 1 C as observed in freshly prepared walls. This procedure was then repeated on two succeeding days with results as illustrated in the figure. The increase in ATP-ase activity observed during the first 48 hours is about 2.5-fold. A similar phenomenon has been observed for muscle ATP-ase (19).

URIDINE DIPHOSPHOGLUCOSE PYROPHOSPHORYLASE. The importance of uridine diphosphoglucose (UDPG) and related uridyl sugars in the biosynthesis of polymeric carbohydrates made a test for the presence of UDPG pyrophosphorylase desirable. This enzyme catalyzes the reaction:

$$UTP + G\text{-}1\text{-}P \rightleftharpoons UDPG + PP.$$

Since cell wall preparations destroyed UDPG (by glucosyl transfer or by simple hydrolysis) and also hydrolyzed UTP, it was convenient to assay this reaction by measuring the G–1–P or UDPG dependent incorporation of P^{32}-labeled PP into UTP. For both soluble protein and cell wall preparations a G–1–P dependent exchange reaction can be observed (table I). In these experiments, the amount of label in nucleotide was determined by adsorption onto charcoal, washing to remove unreacted P^{32}-PP, and measuring the radioactivity of the charcoal (8). Elution of the charcoal with ethanol-NH_3 followed by electrophoresis of the eluates, showed UTP to be the only labeled substance present. Additional experiments demonstrated the formation of labeled UTP in incubation mixtures containing UDPG, P^{32}-PP and unlabeled UTP.

Data from an experiment to determine the distribution of UDPG pyrophosphorylase in the cellular fractions are shown in table II. Note that the presence of enzymes hydrolyzing UTP, UDPG, and PP would all reduce the observed reaction rates so that the reported results are not strictly quantitative. Nonetheless, since to some extent the same factors would probably affect all the preparations tested, one may conclude that the amount of UDPG pyrophosphorylase in cell wall and in soluble protein is of the same order of magnitude, and, that little activity is found in the particulate fraction.

TABLE II

INCORPORATION OF P^{32}-PP INTO UTP*

ENZYME PREPARATION**	REACTION TIME (min)	RADIOACTIVITY IN UTP (cpm/mg N)
Coleoptile homogenate	0.5	270
	5	1,050
	20	2,400
Particulate matter	0.5	120
	5	470
	20	1,000
Soluble protein	0.5	300
	5	4,500
	20	6,600
Cell wall	0.5	2,300
	5	6,000
	20	17,000

* Each tube contained in μmoles: tris 15, ph 7.5; UTP 2, pH 7.5; G–1–P 10: P^{32}-PP 2; $MgCl_2$ 5.

** Enzymes were prepared in 0.01 M tris-0.001 M EDTA, pH 7.5. The total N in the fractions as isolated from 25 g of tissue was 70, 3, 6, and 1 mg, respectively, for the homogenate, particulate matter, soluble protein, and cell walls. Soluble protein was precipitated from solution at 85 % saturation with ammonium sulfate following removal of cell walls and particulate matter.

INVERTASE ACTIVITY. A reaction mixture containing 2 μmoles C^{14}-sucrose, 15 μmoles of tris, pH 7.5, 5 μmoles of $MgCl_2$, and 0.3 mg cell wall protein or 1.85 mg soluble protein was incubated at 37 C for 2 hours, the reaction terminated by boiling for 2 minutes, and the residue sedimented by centrifuging. Aliquots of 0.2 ml of the supernatant fluid were removed for glucose determination by the glucose oxidase method (32). The activities of the cell wall and soluble protein fractions were, respectively, 8.9 and 0.87 μg sucrose hydrolyzed/minute/mg protein^{-1}. Chromatography of aliquots of the reaction mixtures indicated glucose and fructose to be the only products.

TABLE I

UDPG PYROPHOSPHORYLASE ACTIVITY ASSOCIATED WITH CELL WALLS & SOLUBLE PROTEIN

REACTION TIME (min)	INCORPORATION OF P^{32}-PP INTO UTP (mμmoles PP/mg ENZYME PROT.)			
	COMPLETE*		MINUS G–1–P	
	CELL WALL	SOLUBLE PROTEIN	CELL WALL	SOLUBLE PROTEIN
0	1.36	0.11	0.67	0.10
5	12.10	1.74	1.10	0.12
20	31.40	4.00	1.88	0.16

* Each tube contained in μmoles: UTP 2; G–1–P, when indicated, 10; P^{32}-PP 2; tris 15, pH 7.5; $MgCl_2$ 5, and 0.3 mg cell wall protein or 7.5 mg soluble protein. Incubation at 37 C in a volume of 1 ml.

TABLE III

UDPG PYROPHOSPHORYLASE ACTIVITY ASSOCIATED WITH CORN COLEOPTILE CELL WALL FRAGMENTS

REACTION TIME (min)	PP INCORPORATED INTO UPP* (%)		
	SOLUBLE PROTEIN	CELL WALL	SOLUBLE PROTEIN TREATED CELL WALLS
0.5	0.25	0.15	0.25
5	6.25	0.65	0.80
20	3.95	2.20	2.15

* Each tube contained in μmoles: tris 15, pH 7.5; UTP 2, pH 7.5; G-1-P 10; P^{32}-PP 2; $MgCl_2$ 5, and 0.3 mg cell wall protein or 1.5 mg soluble protein.

ENZYME ADSORPTION BY CELL WALL FRAGMENTS. We conducted several experiments to determine the extent to which non-specific enzyme adsorption could account for the enzymatic activity associated with the cell wall fraction. For the experiment reported in table III, cell walls we prepared by the usual method and assayed for UDPG pyrophosphorylase activity. Then we resuspended an aliquot of the wall preparation for 1 hour in 80 % glycerol to which soluble UDPG pyrophosphorylase in an amount equivalent to that present in the original homogenate was added. These conditions approximated those obtained during the initial homogenization of the coleoptile tissue. The cell walls were subsequently reisolated by the usual procedure. As can be seen, we observed no increase in enzymatic activity of the wall fragments, thus demonstrating that additional enzyme was not adsorbed by the wall. There remains the possibility that further adsorption of enzyme was precluded by saturation of the cell wall with enzyme during the initial homogenization.

In an additional experiment, (table IV) we tested the ability of washed, oven dried cell wall fragments to absorb ATP-ase. The heated wall fraction itself inactive, was resuspended as above in 80 % glycerol containing soluble protein of an equivalent amount of tissue. After reisolating the walls by the usual procedure, only 1/20 of the ATP-ase activity found in fresh cell walls was found adsorbed on the heated walls.

Further data bearing on non-specific protein binding as affecting the activities found in the wall fraction were provided by experiments in which the glycerol extracted walls were repeatedly washed in aqueous buffer. As shown by the experiment presented in table V, 60 % of the initial activity remains after six suspensions and resedimentations from a volume of buffer equal to three times the cell wall volume.

TABLE IV

ATP-ASE ACTIVITY IN OVEN DRIED & SOLUBLE PROTEIN TREATED CELL WALLS AS COMPARED TO THAT OF FRESHLY PREPARED & OF OVEN DRIED CELL WALL PREPARATIONS

REACTION TIME (min)	Pi FORMED (μmoles/REACTION TUBE*)		
	CELL WALL FRESH	CELL WALL OVEN DRIED	CELL WALL OVEN DRIED & SOLUBLE PROTEIN TREATED
0	0	0	0
40	3.76	0	0.30
100	7.88	0	0.40

* Each tube contained in μmoles: tris 15, pH 7.5; ATP 5, pH 7.5; $MgCl_2$ 5, and 7.5 mg of cell wall (dry wt) containing 45 μg of N. Cell walls, where indicated, were dried at 80 C for 24 hours.

TABLE V

EFFECT OF REPEATED WASHINGS OF CELL WALL PREPARATIONS ON THEIR ATP-ASE ACTIVITIES

REACTION TIME (min)	Pi FORMED (μmoles/REACTION TUBE*)		
	CELL WALL NOT WASHED	CELL WALL ONCE WASHED	CELL WALL TWICE WASHED
0	0	0	0
10	0.77	0.67	0.61
40	2.99	2.51	2.00
100	6.23	5.39	3.85

* Each tube contained in μmoles: tris 15, pH 7.5; ATP 5, pH 7.5; $MgCl_2$ 5, and 7.5 mg of cell wall (dry wt) containing 45 μg of N. Each washing consisted of suspending and sedimenting the walls three times in a volume of tris-EDTA buffer equal to three times the cell wall volume.

DISCUSSION

Studies of the enzymatic activities of sub-cellular structures become possible after a method for the isolation of such particles in quantity is available. Satisfactory methods for the isolation in quantity of cell walls from higher plants have not previously been available. Some of the difficulties of preparing cell walls are the following. There is, first, the problem of breaking cells completely so that no intact cells survive; second, owing to the intimate connections between the cytoplasmic membrane and cell wall, it may be impossible to obtain preparations free of cytoplasmic membrane remnants and plasmodesmata; third, the isolation of walls free of particulate cytoplasmic inclusions constitutes a serious problem; and last, the possibilities of adsorption of cytoplasmic enzymes to the wall and, conversely, elution of enzymes from the wall poses difficulties. We have previously reported attempts to overcome some of these difficulties in preparing cell wall fragments from corn coleoptile tissue (20). Breakage of cells was attained by high speed homogenization with glass beads in a

glycerol medium; isolation of cell wall fragments was achieved by filtration through a continually renewable filter. The plasmolysis which would occur in glycerol should minimize cytoplasmic membrane contamination, and extensive washing with glycerol would be expected to remove cytoplasmic proteins. The possibility of contamination by these fractions, and especially by the plasmodesmata is, however, difficult to exclude.

Cell wall preparations from 5 day old corn coleoptile tissue showed an appreciable enzymatic activity compared on a nitrogen basis with the soluble protein and particulate fractions from the same tissue. The specific activities of the cell wall UDPG pyrophosphorylase, ATP-ase and invertase were respectively 10, 1 to 2, and 10 times the specific activities of the soluble protein.

The hgher specific activities of cell wall enzymes as compared to cytoplasmic protein would appear to rule out non-specific adsorption of cytoplasmic protein. Such adsorption, under different conditions, has been recently reported by Jansen et al. (18).

Three additional lines of evidence indicated a low adsorption of enzymes to the cell wall. First, no increased UDPG pyrophosphorylase activity was associated with cell walls which had been suspended in a solution of this enzyme and then reisolated. Second, it was shown that oven-heated cell walls, when suspended with equivalent amounts of soluble protein, adsorbed only 1/10 to 1/20 of the ATP-ase activity found in the original cell wall preparation. Third, repeated washing of cell walls with aqueous buffer showed that most of the ATP-ase activity remained with the walls.

Many studies (discussed in the Introduction) suggest that cell wall growth may involve synthetic enzymes located in the wall itself. Previous reports from other laboratories and the present studies have dealt with the enzymatic activities of the wall. It remains unanswered whether enzymes are located in the cell wall proper or attached to adhering cytoplasmic membranes or the plasmodesmata which penetrate the plant cell wall.

Summary

The cell walls of corn coleoptiles prepared in glycerol media showed considerable enzymatic activity as compared with that of the soluble protein and particulate matter from the same tissue. ATP-ase specific activity was approximately as high in the cell wall as in soluble protein and two to three times higher than in other particulate fractions. The specific activity of cell wall invertase and UDPG pyrophosphorylase was approximately ten times higher than that of the soluble protein. Inorganic pyrophosphatase and α-glycerophosphatase activities also were associated with cell wall preparations. We report indications that non-specific adsorption of cytoplasmic enzymes was not an appreciable factor.

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