AUTOLYSIS OF PLANT CELL WALLS IN VITRO

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SU-HWA LEE

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ABSTRACT

AUTOLYSIS OF PLANT CELL WALLS IN VITRO

by Su-hwa Lee

A prerequisite for elongation of plant cells is softening of the rigid plant cell wall. This study was initiated to see if autolysis could be attained *in vitro* in the belief that autolysis may be related to softening. Using a method developed earlier for the isolation of pure primary cell walls of corn (Zea mays) coleoptile tissue, it was found that the walls isolated by a modification of this method contain bydrolytic enzymes. This finding of autolysis of plant cell wall *in vitro* has not been previously reported.

During an eight hours incubation in water at 37° and pH 6.5, about ten per cent of the cell wall is rendered soluble, appearing in solution as reducing sugar and an as yet unidentified partially dialyzable glucose polymer. Glucose appears to be the sole monomeric product of autolysis, but amounts only to one per cent of the initial cell wall weight, or approximately ten per cent of the weight of the solublized portion. It is of interest that twenty per cent of the initially soluble polymer becomes an insoluble white flocculent precipitate on freezing and thawing--that is, it retrogrades. The polymer(s) in many

respects resemble degradation products of callose. Further studies of their physical and chemical properties are needed.

Since invertase is known to be associated with the cell wall, this enzyme was used as a criteria for the general enzymatic activity of the cell wall preparations under study. Most preparations were capable of hydrolyzing 0.025 μmoles of sucrose per hour per mg of cell wall.

The amount of reducing sugar released by autolysis, increased linearly with incubation time and with the amount of cell wall material used. The release of polymer follows the same pattern. Boiling the cell wall preparation before incubation reduces the autolysis by a factor of three to six.

In studies of the pH optimum for autolysis phosphate, acetate and tris buffers were used. Acetate or phosphate ion apparently have some interaction during the incubation period. For eacmple, phosphate buffer increased by almost twofold the amount of reducing sugar released, as compared to a distilled water control at about the same pH (Table 3). The pH optimum curve, which has maxima at 5.5 to 6.5 might be interpreted as the result of effects of acetate and phosphate buffer. It is also possible that the autolysis of cell walls involves more than one enzyme as suggested by the double optimum with respect to pH.

Cell walls prepared from whole coleoptiles showed higher autolytic activity than those from decapitated coleoptiles. This preliminary finding suggests a correlation between autolytic capacity and capacity for elongation growth.

AUTOLYSIS OF PLANT CELL WALLS IN VITRO

By

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INTRODUCTION

As early as 1931, Heyn proposed a possible mechanism for extension growth which involved an increase in cell wall plasticity. Tagawa and Bonner (1957) proposed that rigidity of the cell wall is a function of the number of calcium bridges present in pectic acid. The role of auxin in loosening the cell wall was thought to be due to the reduction of calcium bridges in pectic acid. However, Cleland (1960) concluded that "the removal of calcium cross linkages is not mediated by auxin and that the calcium bridge hypothesis is incorrect." Actually, a large body of evidence indicates that auxin-induced growth in various plant tissues is accompanied by changes in both the cell wall and the cytoplasm, and in nucleic acid matabolism. For example, Liao and Hamilton (1966), employing an autoradiographic technique, have demonstrated in Allium cernuum that the IAA-C^{14} is localized in the nucleus. Thus the mechansim of action of IAA is still unknown, except that the primary site of action may be the nucleus and following that a vast chain of events including cell wall softening is initiated.

Results of several intensive investigations demonstrate that the plasticity of the cell wall is an important factor in cell enlargement. Of major interest is a mechanism which

will account for the alterations in wall plasticity associated with growth. Speculating on the way in which plasticity of the wall could be increased, Frey-Wyssling (1950) proposed that the cutting or dissolving away of the fiber reticulum could bring parts of the wall into a semi-liquid state, allowing the remainder of the reticulum to extend under the turgor pressure of the protoplast. Thus, although we are uncertain as to the mechanism, one of the prerequisites of growth is elongation or softening of the primary wall.

The cell wall develops as plants are growing. In bacteria, fungi or higher plants, disappearance or softening or degradation of the cell walls is involved at different stages in different tissues. The possibility of an autolytic breakdown has been considered in bacterial or fungal cell walls (Mitchell and Moyle, 1957; Arima et al., 1965). In vivo, isotope feeding experiments have indicated that similar processes occur in higher plants (Maclachlan and Young, 1962 and Maclachland and Duda, 1965). The aim of this study was to investigate the softening process of the primary plant cell wall by enzymatic autolysis in vitro.

First, highly purified corn coleoptile cell wall was prepared by the method of Kivilaan et al. (1959), as diagramed in Figure 1. Cell wall preparations were washed free of glycerol with absolute alcohol, acetone and ether at low temperature resulting in a white amorphous powder. Thus the wall preparation utilized was made exactly as

described by Kivilaan et al., except that glycerol was removed with solvents at temperatures of -10 to -20°. This material, designated as cell wall was suspended in buffers or distilled water. and incubated. Autolytic weight losses and the products of autolysis were determined.

The amounts of reducing sugars were determined colorimetrically, sugars were identified by chromatography, and weight losses were determined by weighing the cell wall material either on an electrobalance or on an analytical balance.

The heat lability of autolysis and its exhibition of a pH optimum at near neutral pH indicate the involvement of a typical enzymatic reaction. It is of interest that cell wall prepared from whole coleoptiles had higher autolytic activity than those prepared from decapitated coleoptiles.

LITERATURE REVIEW

Breakdown of the Plant Cell Walls

Neumüller (1958) stated that polysaccharides serving as structural elements in plants could not be broken down by enzymes of endogenous origin, but anatomical evidences have indicated that this is not true. Easu (1953), for example, has cited several instances which may be interpreted as digestion of cell wall material *in situ*. This is especially true, for the primary cell wall at the growing and expanding stages of the cells.

Elongation and Softening of the Primary Cell Wall

That auxin, indolyl-3-acetic acid (IAA) increases the rate of cell elongation in *Avena* coleoptiles by causing the cell walls to become more easily stretchable and more plastic was first suggested by Heyn (1931). This theory lost popularity for a time but in the late 1950's regained it again, as, for example, in the studies of Tagawa and Bonner (1957), employing a "coleoptile bending machine." In their experiments, IAA and potassium ion were able to increase the deformability by increasing the plastic component, whereas calcium ion reversed these effects. Plastic extension of the primary cell wall in enlarging plant cells is thought to require relaxation of the interwoven structure

of the pre-existing wall as well as deposition of new wall material (Setterfield and Bayley, 1961). It is possible that relaxation of the old wall which has usually been attributed to fiber-orientation, could also be facilitated by the breakage of bonds. That bond breakage and "plastic flow" is involved has been demonstrated by Lockhart (1966 and personal communication). With sections excised from the third internode of 8-day-old etiolated pea epicotyls, Maclachlan and Young (1962) were able to show in reality that wall catabolism and wall anabolism are correlated with growth. Their data lead to the conclusion that about one-third of the original insoluble material is dissolved during the time period of their experiment. They proposed that an extensive breakdown of pre-existing wall, rather than the synthesis of new wall, should be regarded as an essential feature of plasticization in enlarging cells. During growth, new wall material is intercalated in amounts which, depending on substrate availability, may or may not replace the material dissolved. Experiments with pollen grains support a relationship between degradation of cell wall material and plasticization of the cell wall. Matchett and Nance (1962) suggested that, when degradation does not result in lysis of the growing tip of the pollen tube, it may cause an increase in plasticity of the pollen tube wall. This can be interpreted as strengthening the argument for the existence of a relationship between degradation of cellular material and plasticity of the primary cell wall.

Dissolution of Secondary Cell Wall

Hartig (1853) and Flach (1924) reported that the dissolution of the end walls was a gradual process originating in one spot in the middle of the wall and spreading from this place to other parts of the wall. Esau (1940), in attempting to determine how continuity is established between vessel segments found that two superposed vessel elements were separated from each other by two cellulose layers. With the electron microscope and shadow cast material, Scott et al. (1960) could not observe the disintegration process and assumed that the end wall during disintegration of the protoplast is broken up into pieces and resorbed. That connection between sieve elements through the sieve areas involved a removal of the cell wall at the pore site was first demonstrated by Esau et al. (1962). The site of the future pores was delimited by the appearance of small deposits of callose. Perforation of the pore site occurs in its center by dissolution of part of the wall and middle lamella, resulting in the union of the callose platelets. Obviously, a variety of enzymatic mechanisms must underlie the highly localized wall differentiation observed in the sieve plate, including the dissolution of the components of the cell wall and middle lamella and the accompanying synthesis of callose.

Autolysis of Bacterial Cell Walls

Autolysis and autolytic enzyme systems have been observed in a variety of Gram-positive and Gram-negative bacteria (Kronish et al., 1964; Mitchell and Moyle, 1957; Murray et al., 1959). A specific autolytic substance has been observed in the supernatant fluids of sporulating cultures of Bacillus terminalis (Greenberg and Halvorson, 1955). This material is relatively heat stable, non-dialyzable, and has a pH optimum in the range of 5.0-5.5. It is precipitated at 0.45-0.70 saturation with ammonium sulfate. The atuolytic system found in Streptococcus faecalis by Shockman et al. (1961) seems to be highly specific. Well-washed walls from cells in the exponential phase will slowly autolyze in phosphate buffer or in distilled water. Lytic activity of the extracts was non-dialyzable and heat-labile. The autolytic system does not seem to be a lysozyme, since the extracts failed to lyse Micrococcus lysodeikticus. Young and Spizizen (1963) also found autolytic enzyme activity in the cell wall of Bacillus subtilis. A heat-labile, non-dialyzable enzyme is present, degrading the cell wall, liberating non-dialyzable hetero-polymers, dialyzable mucopeptides, amino acids and glucose, and an insoluble residue. The non-dialyzable fraction, which constituted 67 to 75 per cent of the cell wall, is composed of at least three heteropolymers with a molecular weight of 15,000 to 20,000.

That these autolytic enzymes play some role in wall growth and cell division has been proposed by a number of laboratories (Mitchell and Moyle, 1957; Shockman et al., 1958; Weidel et al., 1960). Shockman (1965) proposed that perhaps existing bonds are broken in the more or less continuous matrix of wall polymers surrounding the cell, so that a new bit of completed precursor can be inserted, thereby lengthening the polymer chain. On the basis of evidence obtained by use of immunoflurescence, Chung et al., 1964; Cole and Hahn, 1962, and Cole, 1964 and May, 1963, concluded that the bands of new wall synthesis might be initiated by such an autolytic system and correspond to the only areas of wall that are susceptible to the autolytic enzyme system.

Autolysis of Fungal Cell Walls

Enzymatic lysis of fungal cell walls by other micro-organisms has been reported for a number of fungi. Horikoshi and Iida (1958) associated lysis of Aspergillus by some Bacillus species with the action of a chitinase. Mitchell and Alexander (1963) demonstrated chitinase and protease activity in bacterial degradation of Fusarium cell wall. With these fungi the source of the lytic enzymes was another micro-organism. Studies on the autolysis of Aspergillus oryzae, Arima et al., (1965) showed that when mycelia are suspended in water and incubated under suitable conditions, proteins, nucleic acids, and sugars are

excreted into the medium as products of hydrolysis. They found that up to 45°, autolysis occurs faster at higher temperatures. Autolysis occurred readily when the mycelia were incubated in water without glucose under anaerobic conditions. Mitchell and Sabar (1966) showed a direct relationship between fungal growth and autolytic activity. The rate of branching which occurs during development of young hyphae of Pythium, suggests that branching points may be sites of maximum lytic activity. However, both mycelial growing tips and cell wall synthesis may equally account for the realtionship between autolysis and growth. Evidence by Lloyd and Lockwood (1966) also suggests that an autolytic mechanism can account for soil mycolysis. When soil was separated from fungal mycelium by membrane filters having pores small enough to prevent passage of intact organisms, living hyphae of several fungi were completely or partially lysed whereas dead hyphae were not lysed. Complete autolysis of living hyphae of Glomerella cingulata or Hemintho-sporium victoriae was induced by exposure to antifungal antibiotics when the hyphae were kept starved.

AUTOLYSIS OF PLANT CELL WALLS IN VITRO

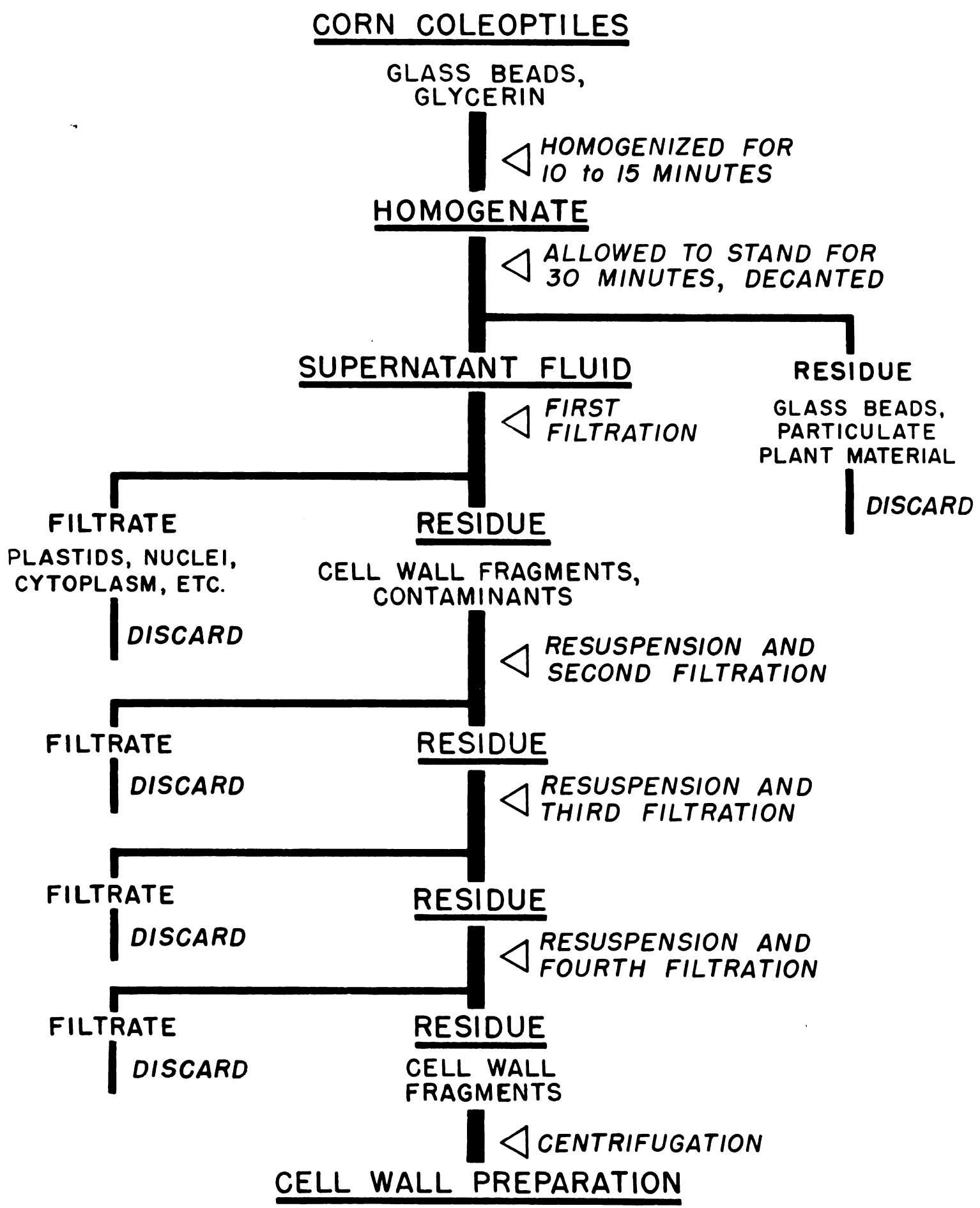
Material and Methods

Preparation of Acetone Powders of the Cell Wall

Glycerol pellet of cell wall preparation.--Cell wall fragments of corn coleoptiles were prepared according to the method previously described by Kivilaan et al., (1959). A summary of the steps involved is given in Figure 1. Michigan 300 hybrid corn (1964) was soaked for 20 hours in tap water. After germination for 4 to 5 days in the dark, coleoptiles were harvested and frozen until used for cell wall preparation. In some cases coleoptiles were decapitated by removing approximately three millimeters of the tip at least five hours prior to harvest. In other cases, whole coleoptiles together with shoot apices were harvested. In such preparations the coleoptile tissue constitutes about two thirds of the total fresh weight. All manipulations were done in a growth room under red light.

Frozen tissue was homogenized in glycerol, repeatedly washed free of subcellular particles and cytoplasm with the same medium and cell wall fragments collected by centrifugation (Figure 1). Cell wall fragments made up five per cent of the final pellet, the rest being glycerol.

Figure 1.--Schematic outline for the isolation of cell wall fragments. Michigan 300 hybrid corn (1964) was soaked in water for 20 hours and germinated for 4 days in the dark. Coleoptiles were harvested and frozen until used for cell wall preparation. Coleoptile tissue (25 gm) was homogenized in a Servall "Omnimixer" for two 5-minute intervals, at 16,000 r.p.m. together with 180 ml of redistilled glycerol and 37 gm of glass beads 200 μ in diameter (Minnesota Mining and Manufacturing Company, Saint Paul, Minnesota).


CORN COLEOPTILES
GLASS BEADS, GLYCERIN
HOMOGENIZED FOR 10 to 15 MINUTES
HOMOGENATE
ALLOWED TO STAND FOR 30 MINUTES, DECANTED
SUPERNATANT FLUID
RESIDUE
FIRST FILTRATION
GLASS BEADS, PARTICULATE PLANT MATERIAL
DISCARD
FILTRATE
PLASTIDS, NUCLEI, CYTOPLASM, ETC.
DISCARD
RESIDUE
CELL WALL FRAGMENTS, CONTAMINANTS
RESUSPENSION AND SECOND FILTRATION
FILTRATE
DISCARD
RESIDUE
RESUSPENSION AND THIRD FILTRATION
FILTRATE
DISCARD
RESIDUE
RESUSPENSION AND FOURTH FILTRATION
FILTRATE
DISCARD
RESIDUE
CELL WALL FRAGMENTS
CENTRIFUGATION
CELL WALL PREPARATION

Preparation of an alcohol-acetone powder from the cell wall glycerol pellet.--Glycerol was removed from cell wall fragments by suspending the pellet in at least one hundred times its weight of cold absolute ethanol, and the cell wall collected by filtration (filter, E-8B Precipitation apparatus, Tracerlab, Boston) on Whatman No. 540 ashless filter paper. Following extensive washing with acetone and peroxide-free ether in the cold, the preparation was recovered and dried overnight under vacuum over phosphorus pentoxide. The resulting white granular powder was stored over anhydrous calcium sulfate at -20°C and used as a cell wall preparation when needed. All procedures were conducted at -5 to -10° and did not require longer than twenty to thirty minutes.

Incubation and Fractionation of Wall Components after Autolysis

In most of the autolysis studies the dry cell wall powder was suspended in glass distilled water (10 mg per ml), a drop of toluene added and the suspension then incubated at 37°. The reaction was stopped by boiling for three to five minutes in a water bath, cooled, the residue collected by filtration under reduced pressure on Whatman No. 540 filter paper, washed with a small amount of water, dried to constant weight, and weight losses determined by a Cahn electrobalance, by an analytical balance, or both. Clear filtrate was kept overnight at -10°, thawed, and the resultant white precipitate formed, collected by centrifugation at 1000 g for five minutes. This procedure was

repeated three times. The pellet so obtained is referred to as non-diahysable polymer or retrogradation product. It was taken to dryness and weight determined with a Cahn electrobalance. Residue of the final supernatant fluid obtained after lyophilization, was taken up in a minimum amount of water, pipetted to weighing pans, redried under vacuum over P_2O_5, and the weight determined by an electrobalance. This is designated as the soluble or dialysable fraction. In cases where glycerol occurred as a contaminant of the dialyzable polymer (see Table 1) it was separated by paper ohromatography and estimated semi-quantitatively by the intensity of the spot of silver nitrate reaction material. Reducing sugars, as soluble autolysis products in the filtrate were determined colorimatrically whereas non-reducing polymers were weighed with the Cahn electrobalance.

In studies of the pH optimum, the acidity of the incubation medium was adjusted from pH 4.0 to pH 5.5 with 0.01 M acetate and 0.01 M K_2H phosphate buffers, from pH 5.5 to pH 7.5 with 0.01 M K_2H phosphate buffer, and from pH 7.5 to pH 10 with 0.01 M tris and 0.01 M K_2H phosphate buffers.

Determination of Total Reducing Sugars and Glucose

Modified Somogyi (1952) and Nelson (1944) methods were employed for the colorimatric determination of total reducing sugar. The copper reagent consisted of solution I (24 gm anhydrous Na_2CO_3; 12 gm $KNaC_4H_4O_6 \cdot H_2O$: 16 gm

TABLE 1.--Autolytic weight loss of cell walls during incubation at 37°C.

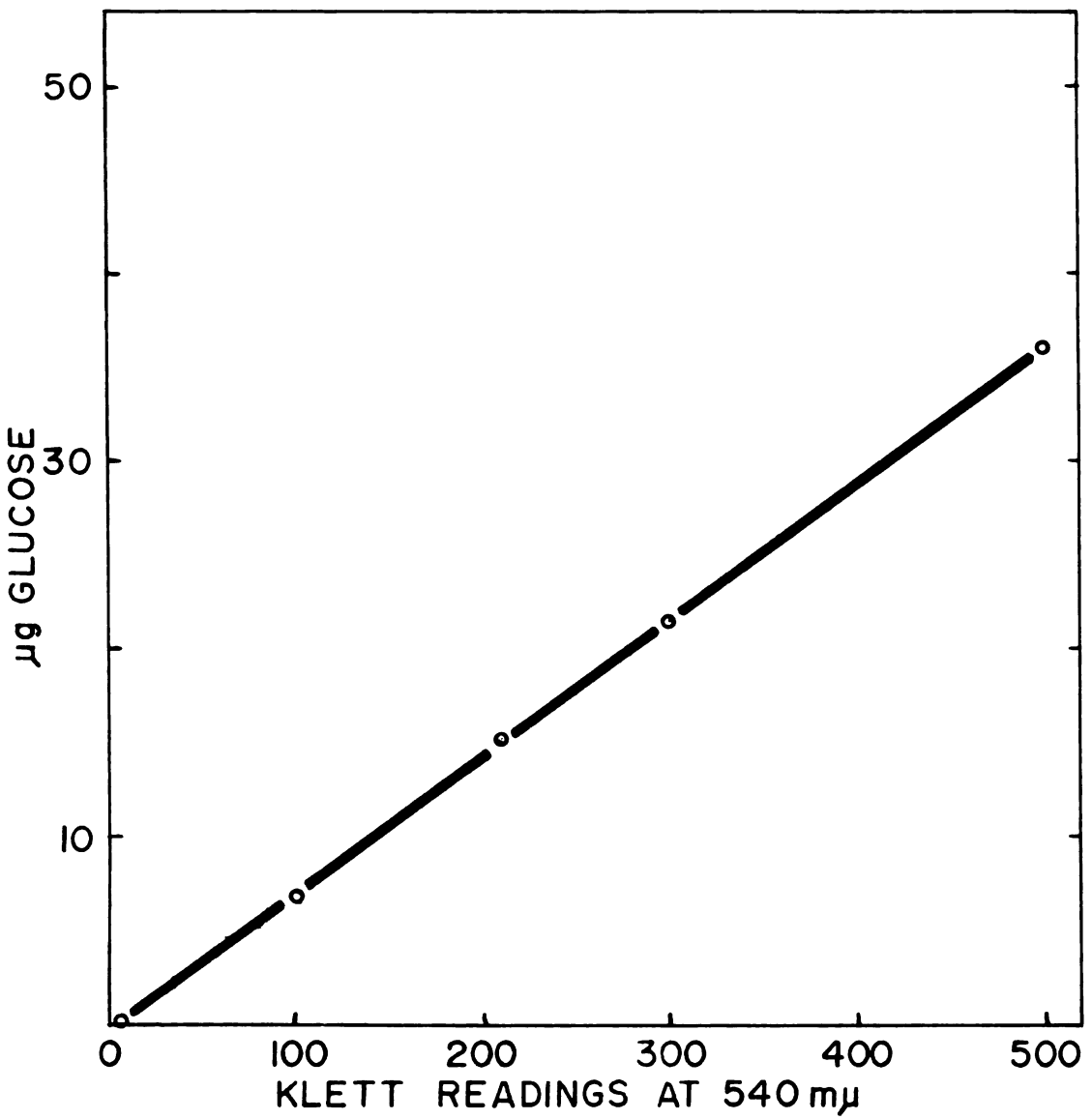
	Boiled after incubation mg	Boiled before incubation mg	Difference mg
Cell wall preparation VII	100	100	
Residue recovered after incubation	80	91	-11
In filtrate:			
Glycerol	7	7	
Reducing sugar	1.2	0.2	1.0
Non-dialyzable polymer	2.6	0.5	2.1
Dialyzable polymer	9.2	1.3	7.9

Incubation was for 8 hours at 37° using 100 mg of cell wall in a total volume of 5 ml. Weight loss of the walls was determined gravimetrically. Glycerol was identified by chromatography and estimated by difference. Reducing sugar was determined by the method of Nelson-Somogyi. Non-dialyzable polymer was estimated gravimetrically after lyophilization of the contents remaining in the dialysis bag. Dialyzable polymer was estimated by lyophilization of the water exterior to the dialysis bag, then extracting glycerol with water, redrying, and weighing the retrograded polymer.

anhydrous $NaHCO_3$; 144 gm anhydrous Na_2SO_4; dissolved in glass distilled water and made to 800 ml), and solution II (4 gm $CuSO_4 \cdot 5\ H_2O$; 36 gm Na_2SO_4; dissolved in 200 ml H_2O). The ratio of solution I to solution II was 4 to 1. The color reagent contained 25 gm $(NH_4)_6Mo_7O_{24}.4\ H_2O$ in 450 ml H_2O; 21 ml concentrated H_2SO_4; 3 gm $Na_2HAsO_4.7\ H_2$). It was "aged," in an incubator at 37°C for 24 to 48 hours and then stored in a glass-stopped brown bottle. The standard curve for glucose concentration was obtained by using a 540 mμ filter in a Klett-Summerson colorimeter and is shown in Figure 2. Glucose was determined by the glucose oxidase method (Saifer and Gerstenfeld, 1958) using the "Glucostat" reagent from Worthington Biochemical Corporation. The solvents used for the chromatography of autolysis hydrolysates were n-butanol : acetic acid : water = 4 : 1 : 5 and ethyl acetate : pyridine: water = 8 : 2 : 1. Sugar spots on chromatograms were detected by aniline-phthalic acid and silver nitrate sprays.

The autolysate filtrate was either applied to the paper immediately after incubation or after storage in a deep freeze. The precipitate which formed upon freezing and thawing was collected by centrifugation and dialyzed against water in dialysis tubing (Union Carbide Corp., Chicago, Illinois) previously washed free of ninhydrin and silver nitrate positive material. The contents of the dialysis tubes and the dialysis water were recovered and taken to dryness by lyophilization. The weight of

Figure 2.--Standard curve for glucose with the Somogyi method. Clucose solution, 0.5 ml, containing between 0 µg to 36 µg were incubated with 0.2 ml copper reagent for 10 minutes in a boiling water bath. The reaction was stopped by cooling and 0.2 ml of Nelson's color reagent added. Absorption was measured employing a Klett-Summerson colorimater with a 540 mµ filter.


50
30
10
µg GLUCOSE
0
100
200
300
400
500
KLETT READINGS AT 540 mµ

non-dialyzable polymer was determined on the Cahn electrobalance, and then partially hydrolyzed in three per cent nitric acid in a sealed tube at 105° overnight and chromatographed. Dialyzable polymer was estimated by lyophilization of the water exterior to the dialysis bag, then extracting glycerol and soluble sugars with water, redrying and weighing the insoluble polymer.

Results

Autolytic Weight Loss of the Cell Wall During Incubation

Cell wall preparations were suspended in glass distilled water and incubated at 37° for 8 hours. The reaction was stopped by boiling in a water bath for 3 to 5 minuted. The average autolytic weight loss of cell wall preparations during an eight hour incubation was approximately ten per cent (Table 1). The loss consisted of reducing sugar, and a partially non-dialyzable polymer, later identified as a glucose polymer.

Products of Cell Wall Autolysis

In filtrates, glucose was the only reducing sugar detected (Figure 3), when chromatographed immediately after filtration. However, on repeated freezing and thawing of the filtrate, a water-insoluble white precipitate was formed. Partial acid hydrolysis of this with three per cent nitric acid at 105° overnight, yielded again only glucose (Figure 4 and Figure 5).

Figure 3.--Diagram of a paper chromatogram of sugars solubilized during autolysis. The filtrate obtained after incubation of cell walls was applied to Whatman No. 1 paper. A pyridine (pyridine : ethylacetate " water = 2 " 8 " 1) was used as developing solvent by the descending technique. Sugars were detected with aniline-phthalic acid. Standard sugars are GAL, galactose; GLU, glucose; FRU, fructose; MAN, mannose; ARA, arabinose; and XYL, xylose.


BOILED AFTER INCUBAT
BOILED AFTER INCUBAT
BOILED BEFORE INCUBAT
STANDARD SUGARS

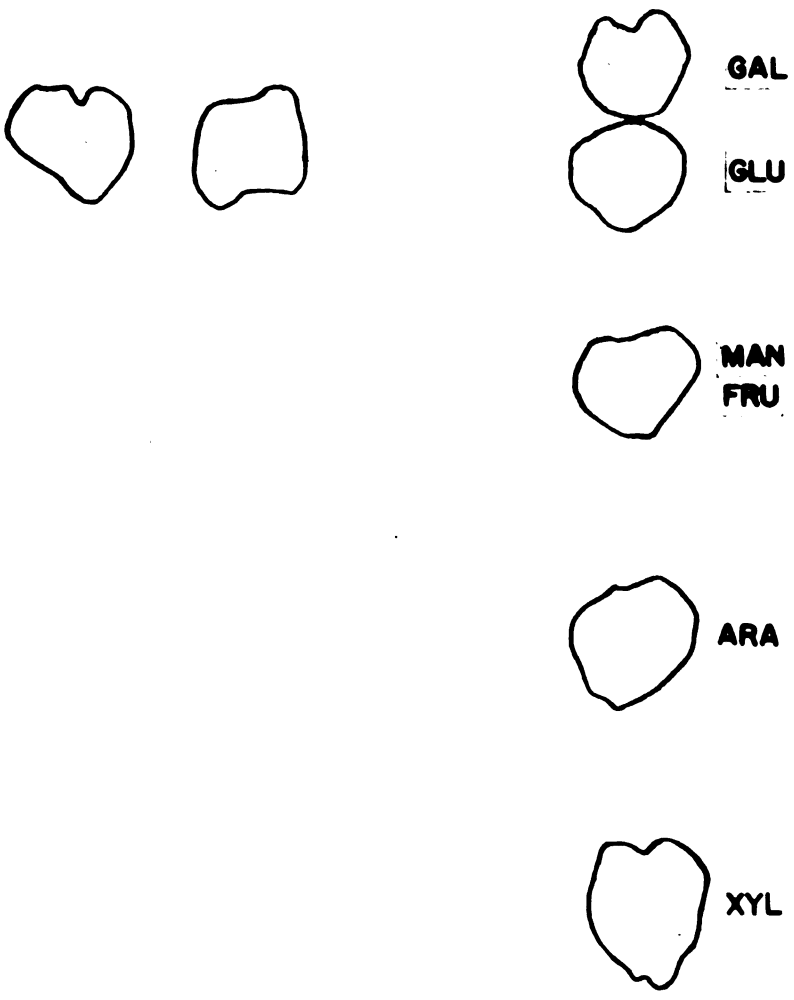

GAL
GLU
MAN
FRU
ARA
XYL

Figure 4.--Diagram of paper chromatography of the hydrolysis products of the dialyzable polymer. Dialyzable fraction of autolysate precipitate after freezing and thawing was chromatogrammed. Hydrolysis as described in the text. Procedure was the same as for Figure 3.

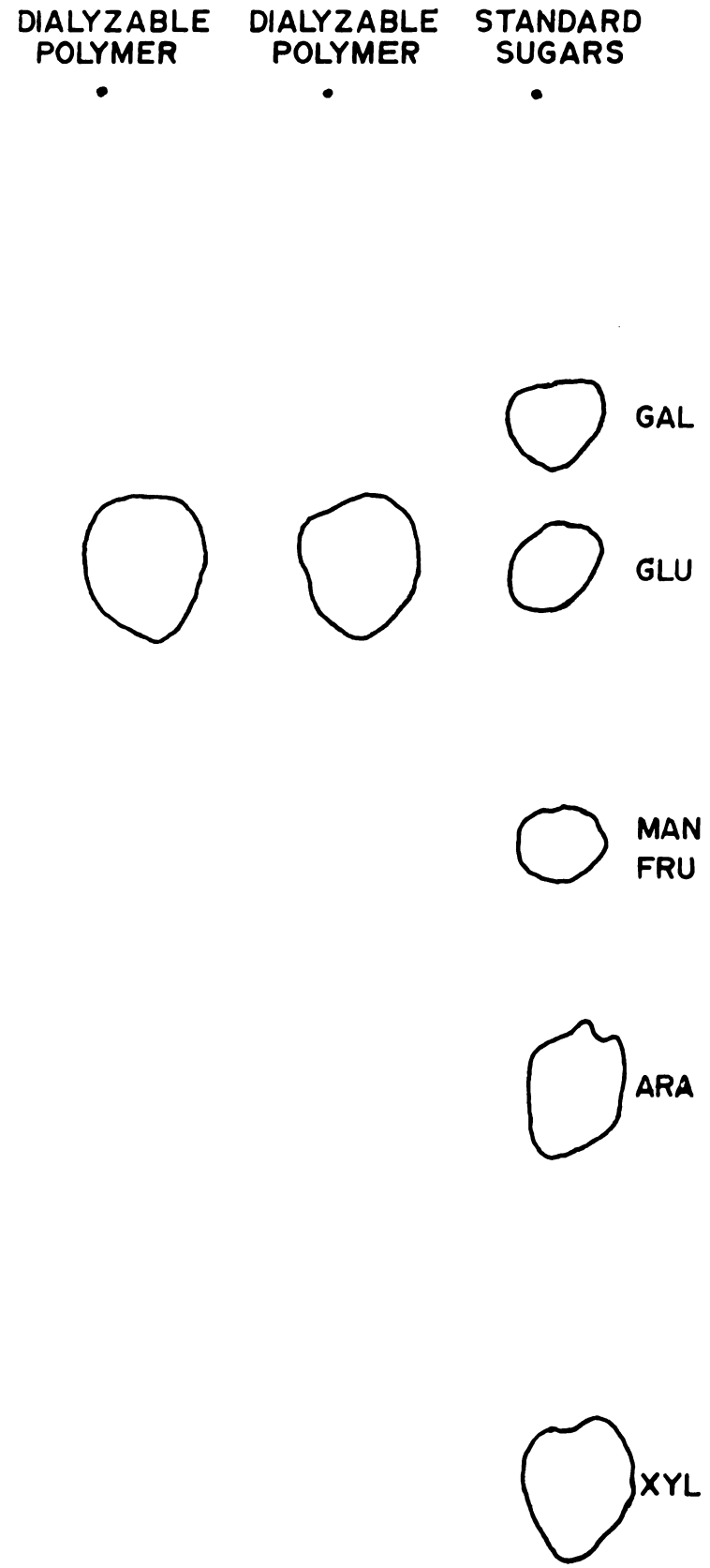
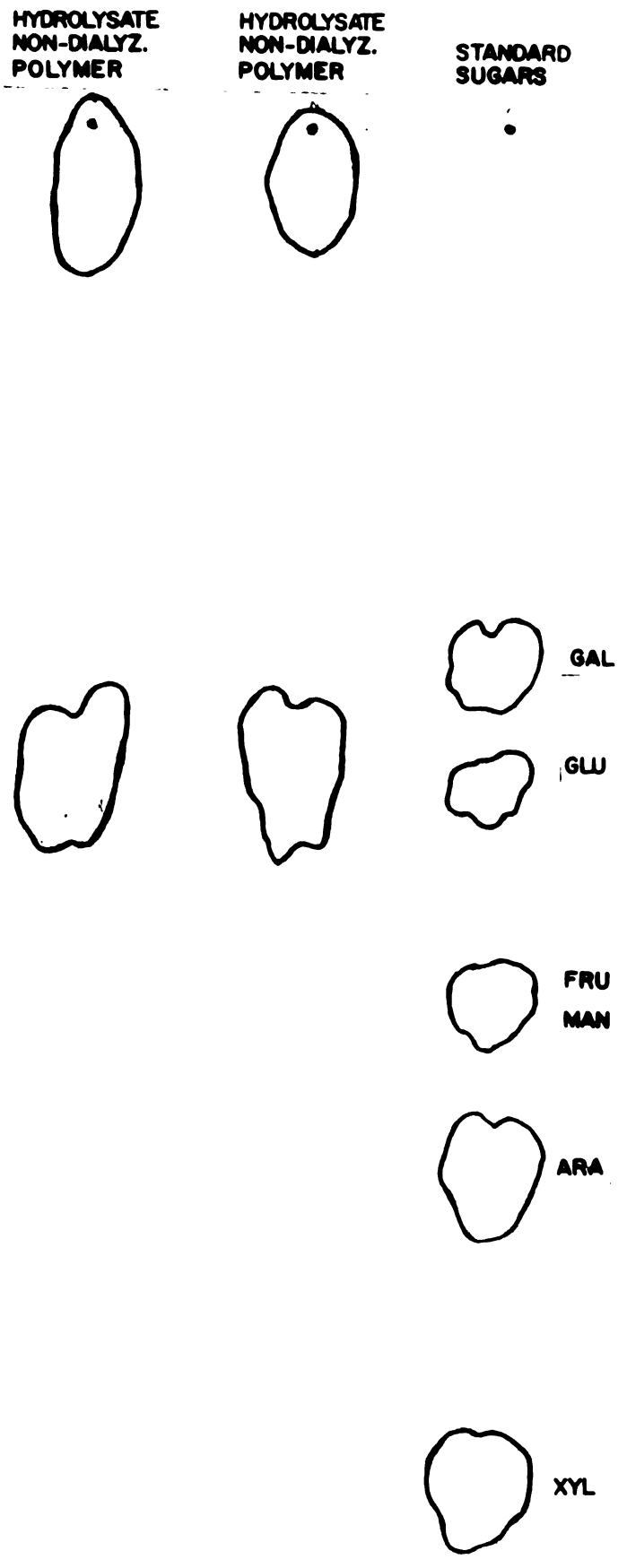

DIALYZABLE POLYMER
DIALYZABLE POLYMER
STANDARD SUGARS
GAL
GLU
MAN
FRU
ARA
XYL

Figure 5.--Diagram of paper chromatography of the non-dialyzable polymer after three per cent nitric acid hydrolysis. Non-dialyzable fraction of autolysate precipitate obtained after freezing and thawing was chromatogrammed. Procedure was the same as Figure 3.


HYDROLYSATE
NON-DIALYZ.
POLYMER
HYDROLYSATE
NON-DIALYZ.
POLYMER
STANDARD
SUGARS
GAL
GLU
FRU
MAN
ARA
XYL

In separate experiments using either 50 mg of cell wall or a commercial α-cellulose preparation (Alphacel of Nutritional Biochemical), the average weight losses of the insoluble residue was determined (Table 2). Alphacel incubated and boiled under the same conditions as the cell wall preparation lost only 5 per cent of its weight compared to 22 per cent weight loss of the cell wall preparation.

Time Course Studies

The enzymatic activity of cell wall acetone powder *in vitro* was studied with time as a variable (Figure 6). The reaction rate was a linear function of time suggested that the release of reducing sugar is a consequence of an enzymatic process and not attributable to microbial contamination.

Effect of pH on Autolysis

Cell wall preparations were autolyzed at pH values between pH 4.0 to 10 during an eight hour incubation period. Incubation was carried out at 37° by using acetic acid buffer (4.0 - 5.5); phosphate buffer (5.5 - 7.5) and tris buffer (7.5 - 10). Equivalent amounts of phosphate were present at all pH levels. Comparing Figure 7 with that of the time course studies (Figure 6), reveals that phosphate buffer increased by almost two-fold (1.9) the amount of reducing sugar released, as compared to a glass distilled water control. It is also shown in Table 3, with a

TABLE 2.--Weight losses of cell walls and alphacel during different treatments.

Treatment of cell wall IX and alphacel	% weight loss in residue
1. Boiled (5 min.); without incubation	5
2. Boiled before incubation (37°C, 8 hours)	9
3. Boiled after incubation	22
4. Incubation without boiling	17
5. Alphacel (Nutritional Biochemical) boiled after incubation	5

50 mg sample of cell wall, or alphacel, was suspended in 5 ml glass distilled water and treated as indicated. The pH of the reaction mixture was 6.8 and did not change during incubation or boiling. Each value is an average of three closely agreeing replicates. Incubation was for 8 hours at 37°. Alphacel was from Nutritional Biochemical Corporation.

Figure 6.--Time course of autolysis of cell wall preparation. A set of tubes containing 50 mg of cell wall preparation were incubated in distilled water (2.5 ml) at 37°C for different time periods, and the filtrates were collected. Reducing sugars were determined by the Nelson-Somogyi method.

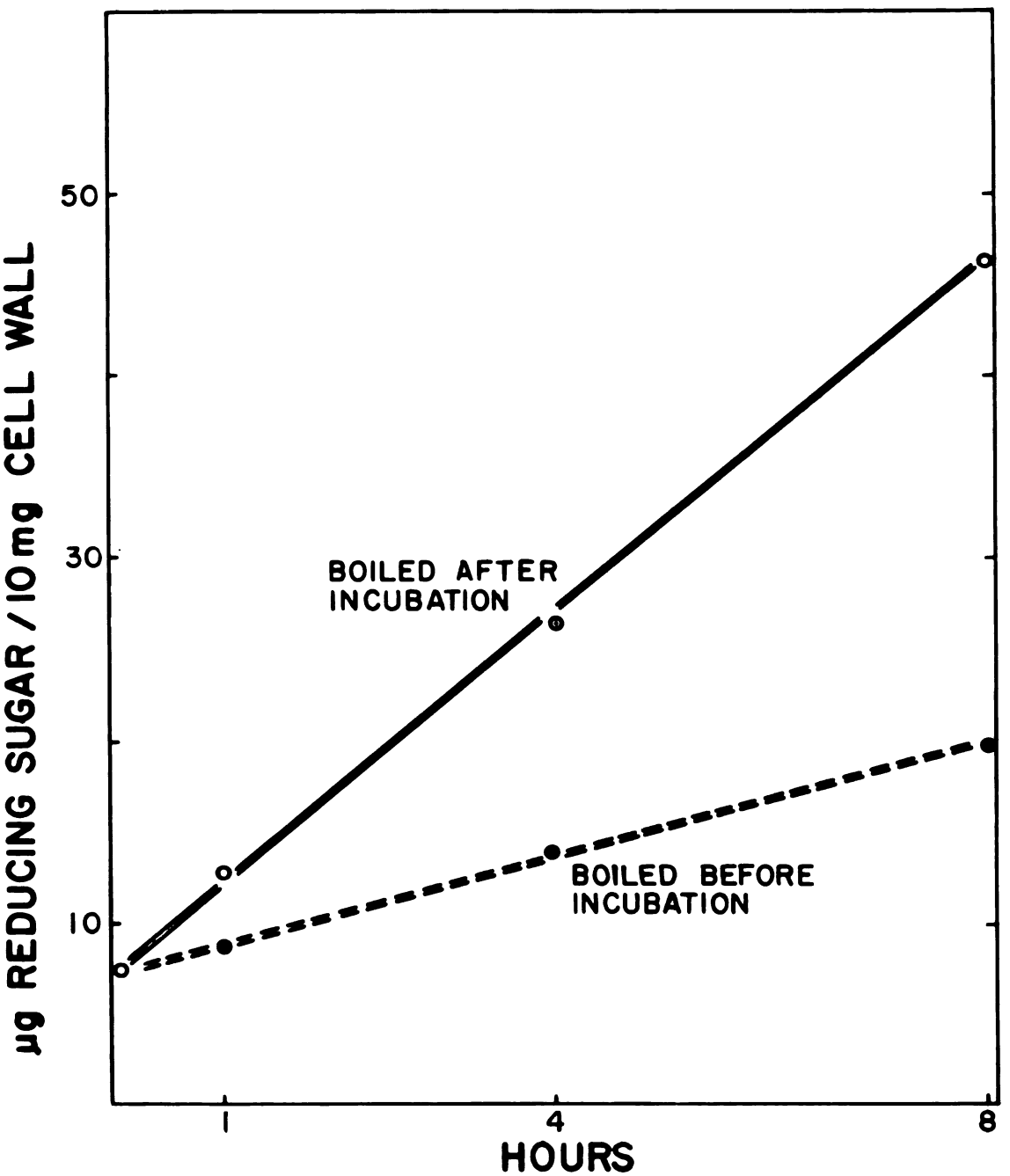

50
30
10
µg REDUCING SUGAR / 10 mg CELL WALL
BOILED AFTER INCUBATION
BOILED BEFORE INCUBATION
1
4
8
HOURS

Figure 7.--Effect of pH on autolysis of cell wall preparations. The assay consisted of 50 mg of cell wall preparation in acetate, dipotassium hydrogen phosphate and tris, all 0.01 molar, total volume of 2.5 ml. Incubation was carried out at 37°C for eight hours.

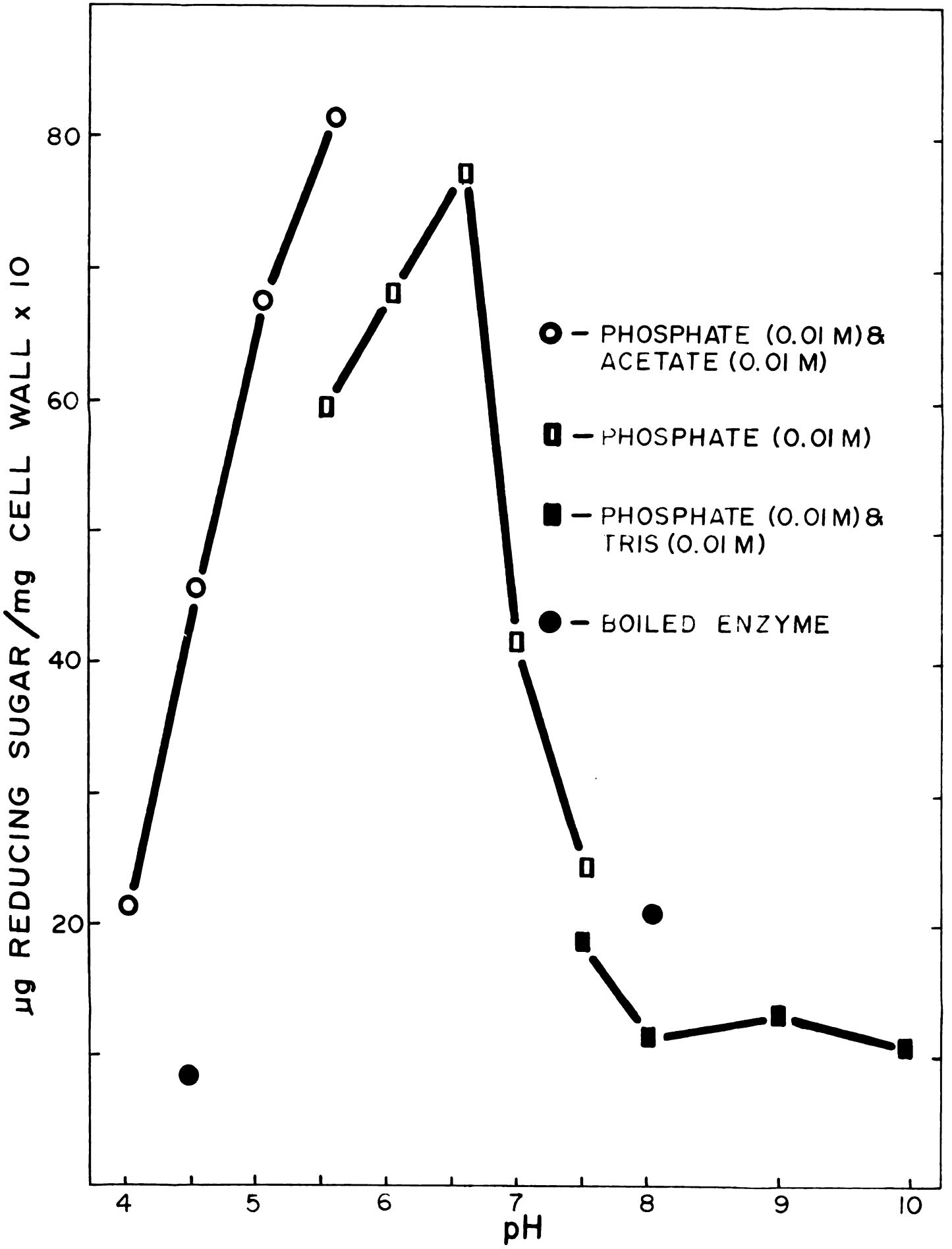

μg REDUCING SUGAR/mg CELL WALL x 10
80
60
40
20
4
5
6
7
8
9
10
pH
PHOSPHATE (0.01 M) & ACETATE (0.01 M)
PHOSPHATE (0.01 M)
PHOSPHATE (0.01 M) & TRIS (0.01 M)
BOILED ENZYME

TABLE 3.--A preliminary single experiment on the effect of potassium, phosphate, and magnesium on cell wall autolysis.

Assay	H_2O	K_2HPO_4 (0.01 M)	$MgCl_2$ (0.001 M)	K_2HPO_4, (0.01 M)	$MgCL_2$ (0.001 M)
Klett units	137	257	85	185	
Reducing sugar	9.6	18.3	6.0	13.0	

20 mg cell wall preparation suspended in 1.5 ml of K_2HPO_4 (0.01 M); $MgCl_2$ (0.001 M); K_2HPO_4 (0.01 M) + $MgCl_2$ (0.001 M); and H_2O for 8 hours incubation at 37 °C.

different incubation medium, that the rate of glucose liberation in phosphate compared to water media is 1.9.

Invertase Activity Associated With the Autolytic System

Sucrose solution (200 µmole sucrose; 1500 µmole Tris pH 8.0; 500 µmole $MgCl_2$ in 100 ml H_2O; final pH 7.6) was used as substrate and incubated with 50 mg of cell wall preparation for 8 hours at 37°. Sucrose hydrolysis was nearly a linear function of time for up to two hours at which time 80 per cent sucrose had been hydrolyzed (Figure 8).

Autolysis of the Cell Wall Preparation as a Function of its Concentration

Different weights of cell wall preparation were incubated with the same amount of glass distilled water at 37° for 8 hours. Autolysis was a linear function of cell wall concentration (Figure 9). It was also found that the precipitate obtained from the filtrate after freezing and thawing was increased approximately linearly as the cell wall concentration increased. In tubes containing only 5 mg of cell wall, there was no precipitate at all; in 10 mg tubes, a barely noticeable precipitate; in 20 mg tubes, noticeable precipitate; in 40 mg tubes, good precipitate; in 70 mg tubes, heavy precipitate, and in 100 mg tubes, a very heavy precipitate. Tubes containing 40 and 70 mg cell wall but having been boiled before incubation showed only

Figure 8.--Cell wall associated invertase activity as a function of time. Incubation of 2.5 me (5 μM) sucrose solution together with 50 mg cell wall for eight hours at 37°C. Reaction was stopped by boiling 3 to 5 minutes and reducing sugar in hydrolysate of sucrose determined by the method of Nelson-Somogyi on Klett-Summerson photometer at 540 mμ.

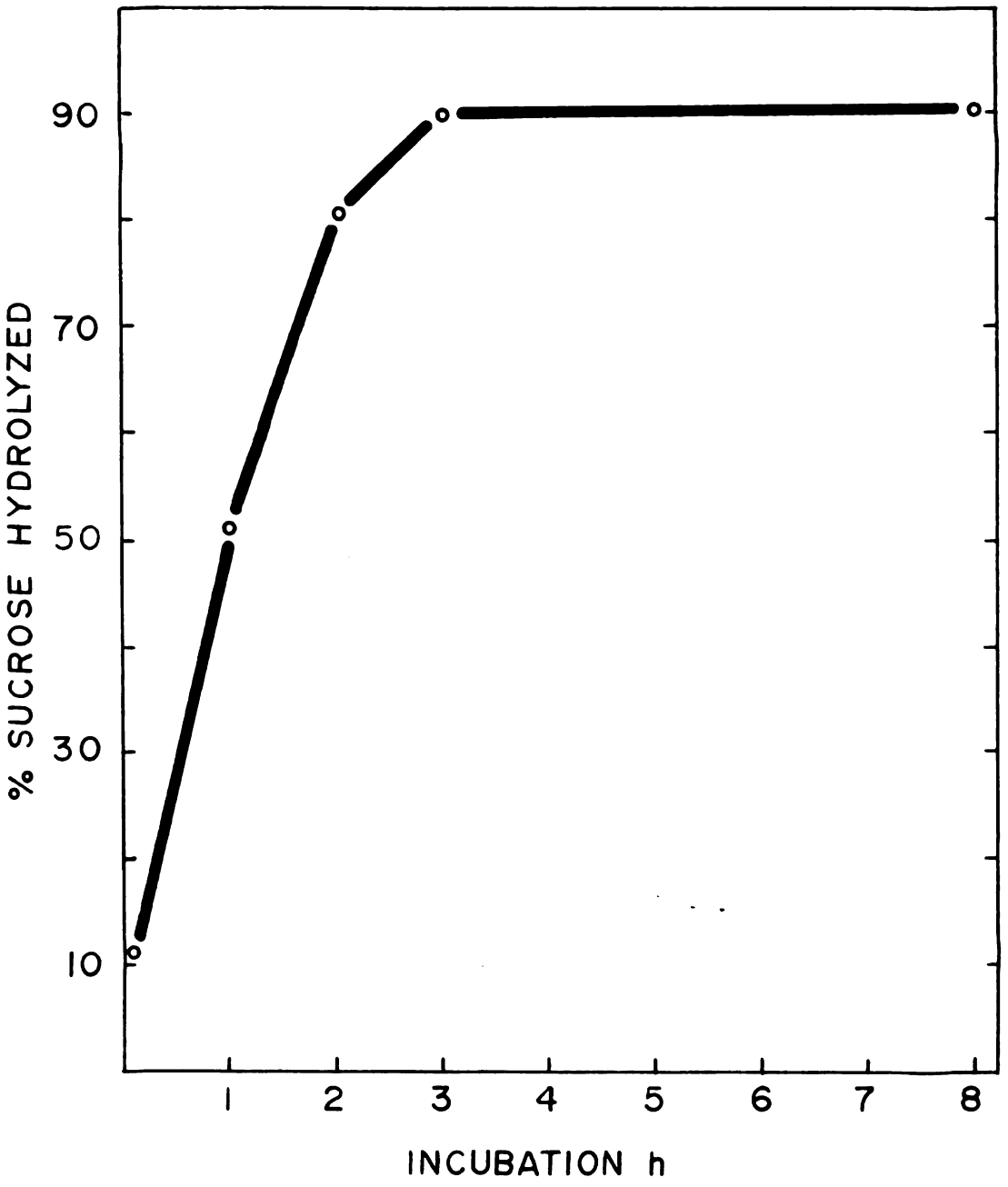
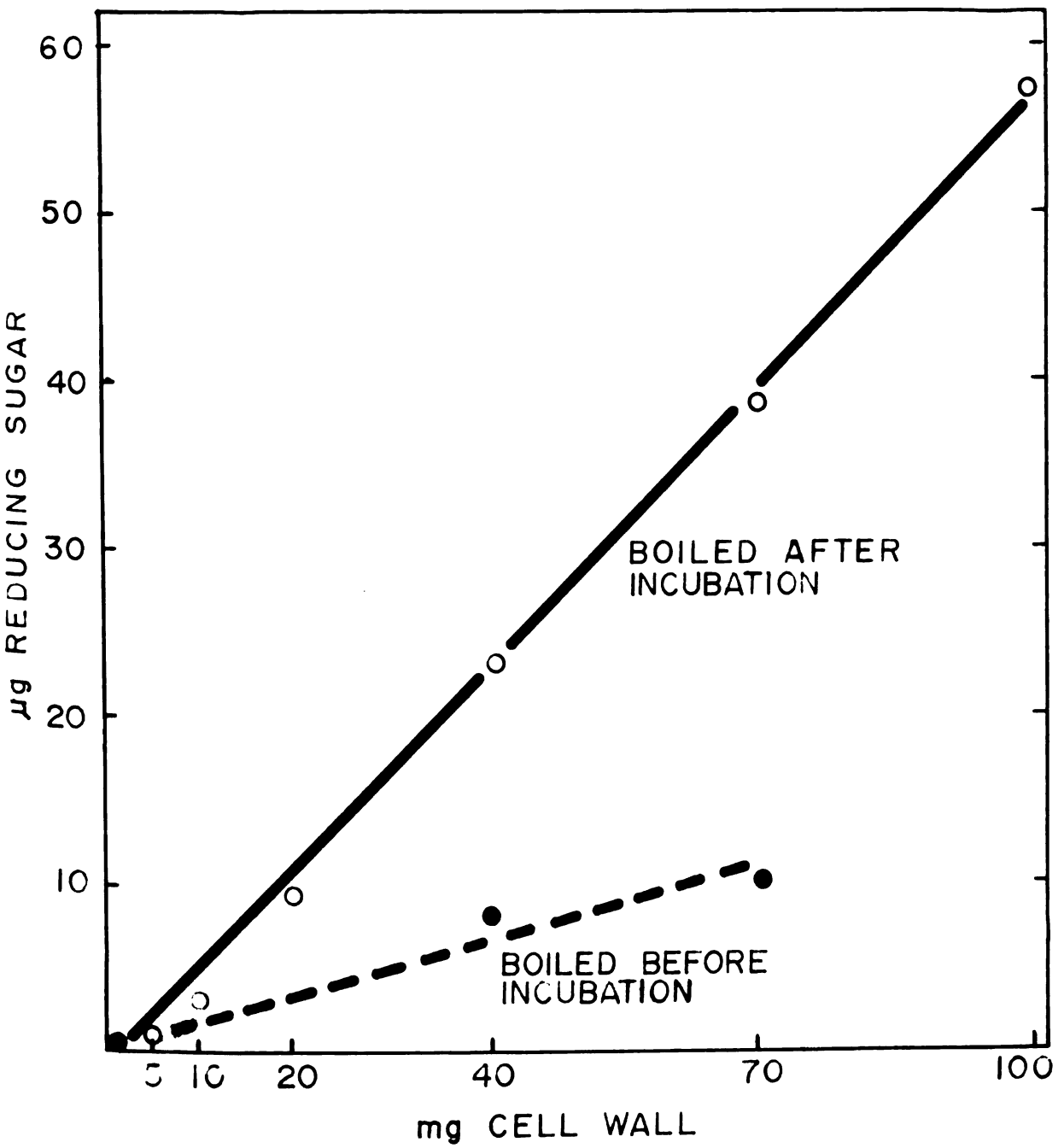

90
70
50
30
10
% SUCROSE HYDROLYZED
1
2
3
4
5
6
7
8
INCUBATION h

Figure 9.--Autolysis as a function of cell wall concentration. 0.5, 10, 20, 40, 70, and 100 mg of cell wall preparations were incubated with 4 ml glass distilled water at 37°C for eight hours. Filtrate was collected, and reducing sugar determined by the Nelson-Somogyi colorimeteric method.


60
50
40
30
20
10
μg REDUCING SUGAR
BOILED AFTER INCUBATION
BOILED BEFORE INCUBATION
5
10
20
40
70
100
mg CELL WALL

a barely noticeable amount of precipitate in filtrates after incubation.

Effect of Indoly-3-Acetic Acid On Autolysis

These experiments are quite incomplete and preliminary but are included owing to their possible interest and help to others. Cell wall (50 mg/tube) was suspended and incubated in 10^{-9}, 10^{-6} or 10^{-3} molar IAA solutions. No significant effect of IAA on release of reducing sugar from the cell wall was observed during the incubation. However, on freezing and thawing of filtrates, the precipitation was noticeably higher in tubes of 10^{-9} and 10^{-6} molar IAA (Table 4).

Effect of Decapitation on Autolysis of Cell Wall

The total amount of reducing sugar released during autolysis was found to be twice as high in cell walls prepared from intact coleoptiles compared to those decapitated prior to harvest (Table 5). It is of interest, that after acid hydrolysis of the filtrate (1 N sulfuric acid for one and half hour in an autoclave), the amount of glucose was found to be equal in both cases.

TABLE 4.--A preliminary single experiment on the effect of Indolyl-3-acetic Acid on cell wall autolysis.

Assay	10^{-9} M IAA	10^{-6} M IAA	10^{-3} M IAA	H_2O
With cell wall (Klett units)	290	256	214	270
Without cell wall (Klett units)	0	0	0	0
Precipitate in filtrate	heavy	heavy	light	light

50 mg cell wall preparation suspended in 2.5 ml of IAA solution and incubated at 37°C for 8 hours.

TABLE 5.--A preliminary single experiment comparing autolytic activity of cell walls prepared from decapitated and intact corn coleoptiles.

Assay	Intact 0°C	Intact 37°C	Δ	Decapitated 0°C	Decapitated 37°C	Δ
Klett units	98	154	56	37	57	20
Reducing sugar (μg)	7.0	11.0	4	2.67	4.06	1.39
Hydrolysis with 1 N H_2SO_4	37°C boiled	37°C		37°C boiled	37°	
Glucose (μg)	6.9	21	14	10	21	11

20 mg cell wall preparation from intact and decapitated coleoptile autolyzed in glass distilled water for 8 hours. Filtrate collected and hydrolyzed with 1 N sufuric acid for one and half hour in an autoclave. Reducing sugar determined by the method of Nelson-Somogyi and glucose by glucose oxidase.

DISCUSSION

The capacity of many surface-active materials to cause lysis of bacterial cultures is a well documented fact. In addition to lytic activity by non-biological detergents, one finds that many biologically produced substances have the ability to destroy the structural integrity of living microorganisms. A number of studies on the autolytic systems of Gram-positive bacterial and fungal cell walls have indicated that a lytic enzyme system can be demonstrated. Data here presented using a cell wall acetone powder clearly demonstrate that breakdown of plant cell walls by autolytic enzyme system also occurs.

In principle there are three general methods for preparing plant cell walls. Osmotic lysis and autolysis might be used to obtain cell wall fragments from unicellular organisms, but mechanical disintegration either by violent agitation with glass beads or sonic and ultrasonic disintegration are still the most commonly used modes of preparation. The only method suitable for disruption of plant cells is mechanical grinding. In the present work corn coleoptile tissue was disintegrated mechanically in glycerol medium to avoid possible elution of cell wall enzymes, and repeatedly washed free of subcellular particles and cytoplasm with the same medium on filters especially prepared

from glass beads (Kivilaan et al., 1959). So, essentially, the cell walls here used were prepared in non-aqueous media in the cold and then freed of glycerol by successive washings with chilled absolute alcohol, acetone and ether. Possibly, the unique method of preparation is responsible for the activity of our cell wall fraction. That the method does possess advantages has been shown by Bean and Ordin (1961).

A function of the autolytic systems has been proposed by Mitchell and Moyle (1957). In this, the lytic enzymes may normally have a synthetic function, in our case, transglucosylation of a cross linkage, like the enzymes synthesizing or degrading the glutamic acid polypeptides of the capsules of Bacillus subtilis and Bacillus anthracis studied by Thorne (1956). Secondly, the lytic enzymes may normally act as hydrolytic elements in the system responsible for the change of shape and disengagement of the cell walls of the dividing bacteria. In either case the activity of the lytic system would be expected to be greater the more rapid the rate of growth. An enzyme hypothesis had been proposed by Shockman (1965) in which the new bit of wall polymer must be inserted into the existing polymeric chain immediately after the action of the potential lytic enzyme at that site. Otherwise, the enzymatic attack on the wall can result in osmotic fragility. Furthermore, the susceptible bond attacked by the enzyme occurs only in the walls

of growing cells. Table 5 shows that under the same condition of incubation, cell walls of intact coleoptiles release more reducing sugar than those of decapitated coleoptiles. This suggests a correlation between total autolytic activity and the rate of growth. After acid hydrolysis, however, the amount of total reducing sugar was the same. This however, is not a difficulty since making the hemi-cellulose matrix less rigid may be of more importance than reducing sugar released. The effect of IAA was not on the hydrolytic product but on the amount of polymer solubilized. This supports the postulate of softening of a hemi-cellulosic matrix. On chromatographic analysis, glucose was the sole product obtained by hydrolysis of the dialyzable or non-dialyzable polymer. According to Bonner (1935), cell elongation is caused by loosening of attachment points that connect the cellulose fibres in longitudinal direction. The loosening of the attachment points could be explained as in the present work by autolytic solubilization of a hemi-cellulose.

As shown in Figure 7, the rate of release of glucose shows a broad, possibly double, pH optima, between pH 5.5 to 6.5. Cellulose, and hemi-cellulose consist of molecules not readily dissolved or degraded unless subjected to relatively high temperature or extremes of acidity or alkalinity. In the laboratory a number of chemical procedures are available for this breakdown, but none of them can compare with the biochemical activities of cellulolytic

micro-organisms. These can completely hydrolyze the most complex forms of undegraded cellulose as found, for example, in cotton fibres, to sugars under mild conditions at about pH 4 to 7. In contrast, adequate chemical procedures cause extensive degradation of all materials present, and produce a grossly contaminated product. The two pH optima in Figure 7 might be explained by saying that the autolytic system of corn coleoptile cell wall involves more than one enzyme. Reese and Mandels (1962) have reported pH 5.5 as the optimum pH value for cellulose.

The products of autolysis are dialyzable glucose, a non-dialyzable polymer, and an insoluble residue which precipitates after freezing and thawing. Partial hydrolysis of the non-dialyzable polymer with nitric acid, yielded glucose as the only product. In germination of pollen, Muller, Stoll and Lerch (1957) found that it was capable of utilizing externally supplied sugar for the synthesis of insoluble polysaccharides. Starch, and β-1, 3-D-glucan, were shown to be present; no evidence was obtained for the synthesis of cellulose or pectin, although these materials are considered to be constituents of the cell wall of pollen tube. The presence of histologically defined callose, in pollen tubes has long been known (Eschrich, 1956). It was demonstrated by methylation analysis that callose from sieve tubes of Vitis vinifera is a β-1, 3-D-glucan (Kessler, 1958). The finding that pollen tube callose is hydrolyzed by a specific β-1, 3-D-glucanase indicates that it is similar in

chemical structure to sieve tube callose. Stability to acid hydrolysis suggests that the non-dialyzable fraction obtained in our preparations might be a β-1, 3-D-glucan. However, more chemical and physical characterization of this glucose polymer is needed for a conclusion.

Wall deformation may occur without cellulosic breakdown, involving instead a slippage of cellulose fibers through the hemi-cellulosic matrix. Nevertheless, it is of interest to consider the cellulases. Cellulose has often been regarded as the skeletal substance irreversibly deposited in the plant cells, and not broken down by enzymes of endogeneous origin (Neumuller, 1958). This may be true for mature plant tisuues. In young cells, however, the breakdown and resynthesis of cellulose in the primary cell wall has been discussed as a possible factor affecting wall plasticity (Matchett and Nance, 1962). Reese and Mandels (1962) tried to detect the activity of cellulase in higher plants. Little or no cellulase was found in the stem and leaves of most of the plants which were examined. If cellulase is involved in the plasticity of growing cells, however, it may be present in all the higher plants at some stages of their development, though in very low concentration only. This may indicate that a general autolytic process releases active proteins from the surface and from internal parts of the cells, or both. Bacterial cellulase seems to be more firmly bound to the cells, or to the cellulose, since culture filtrates of cellulolytic bacteria do not always

contain cellulase (Norkrans, 1963). However, cellulolytic activity can be obtained by treatment with autolytic agents, though even such treatment did not release cellulase from Cytophaga cultures (Fahraeus, 1958). In our studies, different concentration of cellulose and cellobiose were incubated with cell wall preparation. Our results showed no significant cellulase and cellobiase activity. However, Beaman (1964) with the glycerol pellet of corn coleoptile cell wall preparation reported the presence of cellobiase. This discrepancy may be owing to a destruction of cellobiase in the wall by the organiz solvents used to remove glycerol.

SUMMARY

A lytic enzyme system was present in cell wall preparations of corn coleoptile tissue. Incubation at 37°C for eight hours rendered approximately ten per cent of the cell wall soluble. The soluble part consisted of glucose and a partially non-dialyzable glucan. The sole product of partial hydrolysis of glucan with three per cent nitric acid for twelve hours at 105°C was glucose.

Release of glucose was a linear function of the time and the amount of cell wall used during an eight hour incubation period.

The lytic enzyme system of the cell wall preparation had a pH optima of 5.5 to 6.5

Phosphate buffer increased the autolytic activity, as measured by release of free glucose by a factor of 1.86 as compared to glass distilled water.

Cell walls prepared from intact coleoptiles showed higher autolytic activity than those from decapitated coleoptiles.

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