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Muralytic Activities of *Ruminococcus albus* 8

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Ruminococcus albus 8 was cultured with isolated alfalfa cell walls as the carbon source. The culture broth was assayed for muralytic enzyme activities. The effect, with respect to the production of such muralytic enzymes, of growing the microorganism on different carbon sources was also investigated. Also, the rates of solubilization and utilization by *R. albus* of individual alfalfa cell wall sugars during a 96-h growth period were examined.

Ruminococcus albus is one of the most actively cellulolytic of the rumen bacteria, producing a number of extracellular cellulases which completely digest the cellulose surrounding colonies in cellulose agar roll tubes (15). It can also digest the cellulose and hemicellulose in alfalfa and grass hays (8). Dehority (5) has shown that a number of rumen bacteria digest various hemicelluloses, with the resulting solubilized sugars fermented in some cases but not in others. Williams and Withers (18) have investigated the inducibility of muralytic enzymes in *R. albus* RUM 5.

We have embarked on research attempting to increase our understanding of the structure of plant cell walls by examining the components solubilized by enzymes purified from *R. albus* culture medium. As a preliminary phase of this program we have utilized various carbohydrate substrates, including alfalfa cell walls (ACW), to determine the complement of muralytic enzymes produced by our particular *R. albus* strain, strain 8. We have coupled to this assessment of wall-degrading activities a study of the solubilization and relative utilization of individual ACW sugars throughout the culture period. Our data show convincingly that, whereas all wall sugars are rapidly rendered soluble in the culture medium, they are consumed by the bacterium (i.e., disappear from the culture) at different rates.

MATERIALS AND METHODS

Organism and cultivation. *R. albus* 8 was isolated from a fistulated alfalfa hay-fed Jersey heifer and cultured anaerobically on a medium composed of chemically defined constituents plus a source of carbohydrate (9, 16). Stocks were carried by daily transfer via syringe of a 1% inoculum into 6 ml of broth containing 0.15% (wt/vol) pebble-milled cellulose (PMC) and incubated at 39°C. A freshly inoculated stock culture can be stored in the refrigerator for as long as 2 months and retain good viability, but requires 2 days of incubation before the first subculture. One-liter cultures on ACW were used to detect the kinds and amounts of enzymes formed by *R. albus* 8, and 12-ml cultures were used in the enzyme induction studies.

Enzyme production. One-liter cultures grown on 0.3% (wt/vol) ACW for 96 h were filtered with suction through an 11-cm Whatman GF/A glass-fiber disk to remove undigested wall material. The filtrate was centrifuged at 25,000 × *g* for 20 min, and the supernatant was passed through a polycarbonate 0.4-μm filter (Nuclepore Corp., Pleasanton, Calif.) to remove remaining bacterial cells. This filtrate was concentrated by ultrafiltration through a PM-10 membrane (nominal molecular weight cutoff of 10,000; Amicon Corp., Lexington, Mass.) to a volume of ca. 20 ml, and aliquots of this retentate were assayed for enzyme activity.

Induction experiments. Enzyme induction studies used basal liquid medium with the following carbon sources, respectively, all at 0.3% (wt/vol): PMC, ACW, β-1,4-xylan from larch, cellobiose, beet araban, polygalacturonic acid, and a uronide-rich preparation derived from ACW (polygalacturonase-soluble carbohydrate [PGSC]; see below). At various times during growth, 1-ml aliquots were removed aseptically and anaerobically and centrifuged for 10 min at 15,000 × *g* in 1.5-ml disposable microcentrifuge tubes (Bio-Rad Laboratories, Richmond, Calif.), using an Eppendorf model 5412 microfuge (Brinkmann Instruments, Inc., Westbury, N.Y.). Samples (0.75 ml) were removed from the supernatant and diluted with an equal volume of buffer (0.1 M HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], pH 6.8, containing 0.001 M $CaCl_2$). Enzymatic activities of the diluted supernatants were assayed in duplicate. Protein soluble in these culture supernatants was measured by the Coomassie blue dye binding method of Bradford (3).

Glycanase activities. Glycanase activities were measured by following the increase in reducing equivalents, using the method of Nelson (13) as modified by Somogyi (14). Assays contained 0.25 ml of 0.1% (wt/vol) substrate solution (see below), 0.15 ml of buffer (0.1 M HEPES–0.001 M $CaCl_2$, pH 6.8), and 0.1 ml of enzyme.

Larch xylan was purchased from Sigma Chemical Co. (St. Louis, Mo.) and purified as described by Taiz and Honigman (17) to obtain the β-1,4-xylan. Cellulose was obtained by rolling 30 g of Whatman no. 1 filter paper for 24 h at 75 rpm in 1 liter of water in a 1-gallon (ca. 3.8-liter) porcelain jar loaded with flint pebbles (Arthur H. Thomas Co., Philadelphia, Pa.) up to the surface of the water. Beet araban (Koch-Light Laboratories, Ltd., Colnbrook, England) and polygalacturonic acid (Sigma) were used without further purification. β-1,4-Galactan was purified from pectin as described by Labavitch et al. (10). One unit of enzyme activity on a polysaccharide substrate is defined as 1 μmol of reducing sugar released per min at 37°C.

Glycosidase activities. Glycosidase activities were determined by measuring the amount of *p*-nitrophenol released from *p*-nitrophenyl glycoside substrates. A typical assay contained 0.5 ml of 10 mM substrate in 0.1 M HEPES, pH 6.8, and was initiated by addition of enzyme. The reaction was terminated by addition of 1.0 ml of 1 M NH_4OH containing 1 mM EDTA, and the *p*-nitrophenol released was measured by the increase in absorbance at 400 nm.

The *p*-nitrophenyl substrates used for glycosidase determinations were α- and β-D-galactoside, α-L-arabinoside, α-

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TABLE 1. Carbohydrate composition of ACW material[a]

Component	Amt (mg) per 18 mg (air dry wt) of ACW	% (by wt)
TFA-soluble neutral sugar		
Rhamnose	0.33	1.8
Fucose	0	0
Arabinose	1.04	6.1
Xylose	1.34	7.4
Mannose	0.18	1.0
Galactose	0.60	3.3
Glucose	0.70	3.9
TFA-soluble uronic acids	3.68	20.4
Cellulose	6.87	38.2

[a] The analysis was performed on the insoluble material collected by centrifugation of an uninoculated (control) culture. The data thus represent the identified carbohydrate components in the ACW originally supplied to the cultures.

and β-D-glucoside, α-D-mannoside, α- and β-D-fucoside, and β-D-xyloside (Sigma) and α-D-xyloside (Koch-Light). One unit of activity equals 1 μmol of substrate hydrolyzed per min at 37°C.

Culture substrates. ACW was prepared from freshly cut alfalfa. A sample of ACW was treated with a purified polygalacturonase (PG) to give uronide-rich wall preparation (PGSC). The preparation of ACW and PGSC is described in detail in the preceding paper (7). The composition of ACW is described in Table 1. The carbohydrate composition of PGSC is 51% (wt/wt) uronic acid, 27% arabinose, 9% galactose, and 2% xylose.

Identification and measurement of carbohydrates metabolized by *R. albus* 8 when grown on ACW. Cultures (12 ml) containing 18 mg of ACW were used to examine the utilization of the individual carbohydrate components of ACW. Growth on ACW is quite rapid. Therefore, the cultures were initiated with only 0.5% inoculum to slow solubilization and use of ACW sugars.

Replicate cultures were inoculated and continuously agitated in a tube roller at 39°C, with an uninoculated tube as a control. Individual tubes were removed at 12-h intervals and immediately centrifuged at 1,000 × *g* for 20 min. The pellet and supernatant were separated and frozen at −15°C for later processing.

TABLE 2. Enzymatic activities assayed in filtrate from cultures of *R. albus* 8 grown for 96 h on ACW

Activity[a]	U/liter
α-Glucopyranosidase	0
β-Glucopyranosidase	13
β-Galactopyranosidase	15
α-Galactopyranosidase	63
α-Arabinofuranosidase	65
α-Mannopyranosidase	0
α-Fucopyranosidase	0
β-Fucopyranosidase	0
β-Xylopyranosidase	7
Cellulase	71
β-1,4-Xylanase	97
PG	79

[a] Activity was measured as micromoles of reducing sugar hydrolyzed per minute. Substrates for glycosidases were *p*-nitrophenyl glycosides, larch xylan for the xylanase, polygalacturonic acid for the PG, and PMC for the cellulase.

The pellets were lyophilized and then hydrolyzed with 1.0 ml of 2 N trifluoroacetic acid (TFA) for 1 h at 121°C. This reaction mixture was centrifuged at 2,000 × *g* for 15 min, and the supernatant was transferred to another tube, dried under a stream of N_2, and resuspended in 1.0 ml of deionized water. One-half of this TFA-soluble material was assayed for uronic acids by the method of Blumenkrantz and Asboe-Hansen (2), and the other half was analyzed for neutral sugars by gas-liquid chromatography according to Albersheim et al. (1). The TFA-insoluble pellet was treated with 67% (vol/vol) sulfuric acid for 12 h at room temperature to dissolve the cellulose, and aliquots from the resulting suspension were analyzed for carbohydrate by the anthrone method (6), calibrated with a standard solution of cellulose powder dissolved in the sulfuric acid solution.

Separate aliquots of the culture supernatants were analyzed for uronic acids (2) and hydrolyzed in 2 N TFA and analyzed for neutral sugars (1).

From the disappearance of components from the culture pellet the rate of solubilization could be calculated, and from the rate of disappearance from the total culture (combined pellet and supernatant fractions) the rate of utilization could be determined. (Bacterial capsules will be recovered in culture pellets. Because these capsules contain some of the sugars present in ACW [glucose, mannose, and rhamnose; R. J. Stack, unpublished data], there is a potential that rates of utilization would be understated. However, we calculate that much less than 1% of any of the sugars measured in the pellets could have come from bacterial material.)

RESULTS

The carbohydrate composition of the ACW is shown in Table 1. Cellulose is the most abundant component, with uronic acids next. The TFA-soluble neutral sugars were, in order of their abundance, xylose, arabinose, glucose, galactose, and mannose. No fucose was detected. The ACW preparation undoubtedly contained some protein and lignin, but these were not measured.

R. albus 8 produces a wide variety of muralytic enzymes when grown on ACW (Table 2). Araban and β-1,4-galactan were the only substrates not digested in our assays. The time course for the production of the prominent enzyme activities

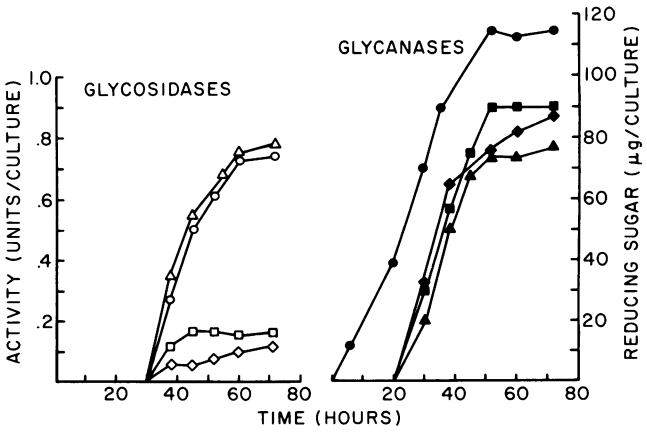

FIG. 1. Time course for the appearance of cell wall-degrading enzymes in the extracellular broth of cultures of *R. albus* 8 grown on ACW. Total reducing sugar of the culture was also measured. Symbols: □, β-glucosidase; ◇, β-xylosidase; △, α-galactosidase; ○, α-arabinosidase; ◆, cellulase; ■, PG; ▲, β-1,4-xylanase; ●, reducing sugar.

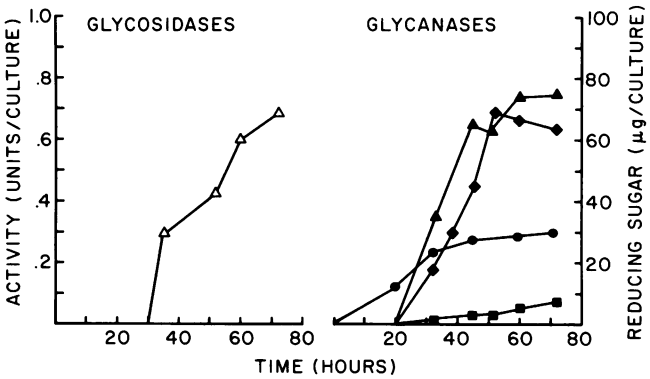

FIG. 2. Time course for the appearance of polysaccharide-degrading enzymes in the extracellular broth of cultures of *R. albus* 8 grown on PMC. Total reducing sugar of the culture was also measured. Symbols: △, α-galactosidase; ◆, cellulase; ■, PG; ▲, β-1,4-xylanase; ●, reducing sugar.

is depicted in Fig. 1. The reducing sugar in the culture increased before significant activity of carbohydrases was detected. The glycanase activities (PG, cellulase, and β-1,4-xylanase) are detected before the glycosidases. Figure 2 shows the activites when *R. albus* 8 is grown in PMC. Reducing sugar generation was much less than in the ACW cultures but once again preceded the appearance of glycanases. Cellulase and β-1,4-xylanase activities were prominent but PG activity (on a culture basis) was only 10% of that in the ACW cultures. The only glycosidase detected was α-galactosidase.

Figure 3 shows that, when *R. albus* 8 is grown on β-1,4-xylan, xylanase is the only detected activity of any consequence. The very small amounts of PG and cellulase detected at 60 and 72 h may represent release of intracellular enzyme or production during the stationary phase of growth. However, in these cultures, as in all others, the accumulation of protein in the culture medium was steady throughout the 96-h observation period (data not shown), suggesting that cell lysis did not lead to the later appearance of these activities. Figure 4 shows the production of enzymes when *R. albus* 8 is grown on polygalacturonic acid, beet araban, PGSC, and cellobiose. No glycanases, including arabanase, could be measured in the araban-grown culture. In spite of this the culture grew (measured by following increase in soluble protein). Arabinosidase was detected in all cultures. β-Xylosidase, which was not found in the xylan cultures, was produced during growth on PGSC. The time frame in which specific enzyme activities developed in the cultures depicted in Fig. 4 was, in general, the same as in cultures grown on ACW, cellulose, and xylan. A notable exception is the relatively early appearance of α-arabinosidase in the PGSC culture (Fig. 4C).

Figure 5 depicts the time course for solubilization of the various sugars in ACW. Inspection shows that each of the wall constituents measured was rapidly made soluble and that at 96 h the insoluble fractions of all components constituted <20% of the total initially presented in the air-dried ACW. The high zero-time values for arabinose, uronic acids, and rhamnose represent the fraction of these materials made soluble during media preparation and sterilization. Gas-liquid chromatography analysis of culture supernatants that had not been acid hydrolyzed indicated that none of the soluble neutral carbohydrate was in monosaccharide form.

The relative utilization of individual ACW sugars is shown in Fig. 6. Glucose, xylose, and mannose were rapidly used by *R. albus*; there was little consumption of these sugars after 24 h even though none of them was totally removed from the culture. In contrast, rhamnose, galactose, and uronic acid were used at a slower, steady rate throughout the culture period. Arabinose was also used at a steady rate, but more rapidly and completely. Glucose was the only sugar showing parallel utilization (Fig. 6) and solubilization (Fig. 5) curves.

Glucose (cellulosic and noncellulosic) and xylose comprise the bulk of the carbohydrate in ACW (Table 2) and were used during the first day of culture (Fig. 6). Table 3, which shows the consumption of carbohydrate in absolute terms, indicates that 75% of the sugar utilized over the 96-h culture period was used in the first 24 h and that over 90% was used in the first 48 h.

DISCUSSION

The enzymes produced by *R. albus* 8 are sufficient to digest most of the autoclaved ACW (Table 3) and are probably capable of solubilizing the bulk of the carbohydrate components of most dicots (10). In addition to the identified glycanases and glycosidases, there may be as yet unidentified enzymatic activities which contribute to the digestion of ACW but were not measured because of lack of information about appropriate substrates.

The effects of various culture substrates on the production of both glycosidase and glycanase enzymes indicate partial regulation of enzyme production by the substrate or products from it. Our observations agree in general with those of Clarke et al. (4) and Williams and Withers (18). Williams and Withers investigated the inducibility of cell wall-degrading enzymes in *R. albus* RUM 5. This strain did not produce α-arabinofuranosidase under any conditions tested. In our *R. albus* cultures cellobiose did not induce the production of appreciable amounts of xylanase (Fig. 4D), whereas it did in RUM 5.

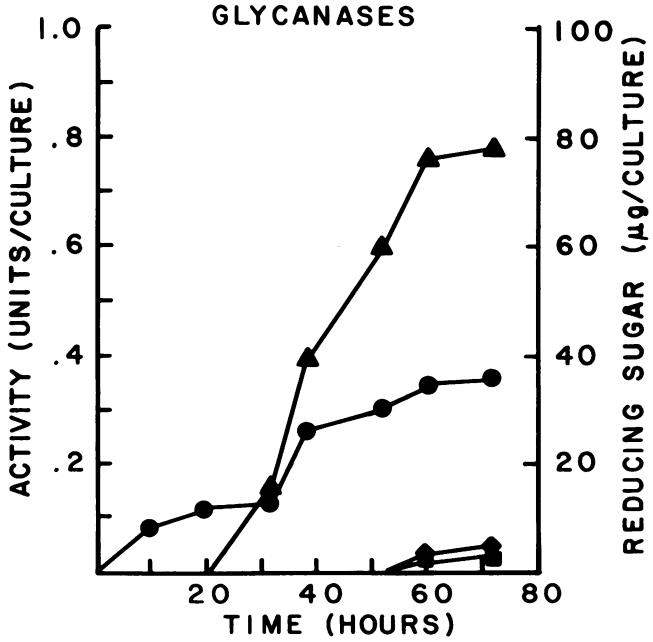

FIG. 3. Time course for the appearance of polysaccharide-degrading enzymes in the extracellular broth of cultures of *R. albus* 8 grown on β-1,4-xylan. Total reducing sugar content of the culture was also measured. Symbols: ▲, β-1,4-xylanase; ●, reducing sugar.

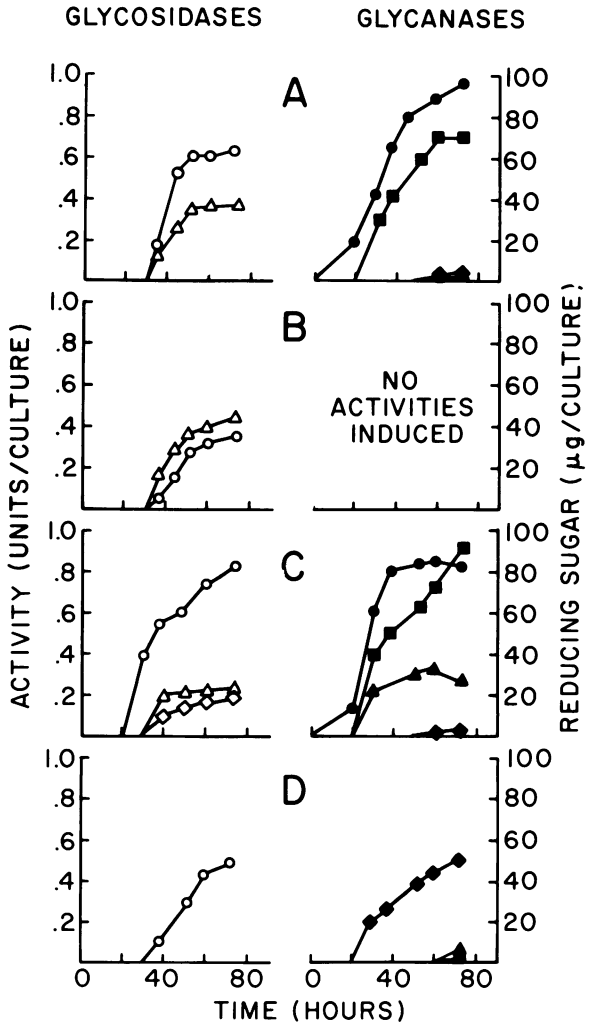

FIG. 4. Time course for the appearance of polysaccharide-degrading enzymes in the extracellular broth of cultures of *R. albus* 8 grown on polygalacturonic acid (A), beet araban (B), PGSC (C), and cellobiose (D). Total reducing sugar of each culture was also measured. Symbols: △, α-galactosidase; ○, α-arabinosidase; ◇, β-xylosidase; ◆, cellulase; ■, PG; ▲, β-1,4-xylanase; ●, reducing sugar.

The activities of the various glycosidases on *p*-nitrophenyl substrates are likely to be different from those on natural substrates. However, the measurement of *p*-nitrophenol is at least as sensitive as the measurement of reducing sugars. Thus, our ability to detect glycosidase activities is somewhat better than our ability to detect glycanases. That glycosidase activities generally rise after glycanase activities indicates separate regulatory systems for these enzyme groups and suggests that products of glycanase action (presumably oligosaccharides) are inducers of glycosidases. It is interesting to note that the one instance of glycosidase activity (α-arabinosidase) increasing concurrently with glycanase activities (culture on PGSC; Fig. 4C) involved a substrate that was itself a glycanase product (albeit polymeric) and which might require α-arabinosidase action for effective PG action (7). An explanation for the appearance of β-xylosidase in cultures on ACW and PGSC but not in xylan cultures (which contain substantial β-1,4-xylanase; Fig. 3) is not readily apparent.

Reducing sugar levels in the culture media begin to rise soon after inoculation (Fig. 1 to 3). It is likely that the amounts of reducing sugar reported, especially during the latter part of the culture period, are a minimum indication of enzyme action because of utilization of soluble carbohydrate by *R. albus*. The increase in reducing sugar before detection of hydrolytic activities is probably due to an initial, almost total adsorption of the enzymes on the insoluble substrates in the sediment that was separated by centrifugation from the supernatant containing the tested enzymes. As digestion of these substrates continued, the enzymes would become soluble and, thus, detectable by our analyses. Because we have not attempted to recover enzymes from the pellet fractions of the cultures, it is possible that our measurements of enzyme activity are lower than the amounts actually present.

The PG activity in the PMC culture (Fig. 2) was not expected, but subsequently we found that PMC prepared from Whatman no. 1 filter paper contains small amounts of uronic acid which could have induced PG activity. The PMC was analyzed for xylose but none was found, making it unlikely that traces of xylose could account for the β-xylosidase activity which developed. Because β-D-xylopyranose has a configuration similar to C-1 to C-5 of β-D-glucopyranose, it may be that some cellulases (β-1,4-glucanases) do not completely discriminate between β-1,4-linked xylopyranosyl and glucopyranosyl residues; if C-6 is not important in the binding or catalytic process, the two sugar moieties might be interchangeable and the cellulase could also act as a xylanase. A β-1,4-xylanase, on the other hand, may have evolved with a binding site incapable of accommodating the C-6 portion of a glucose residue. This distinction may account for the lack of cellulase activity in the xylan cultures.

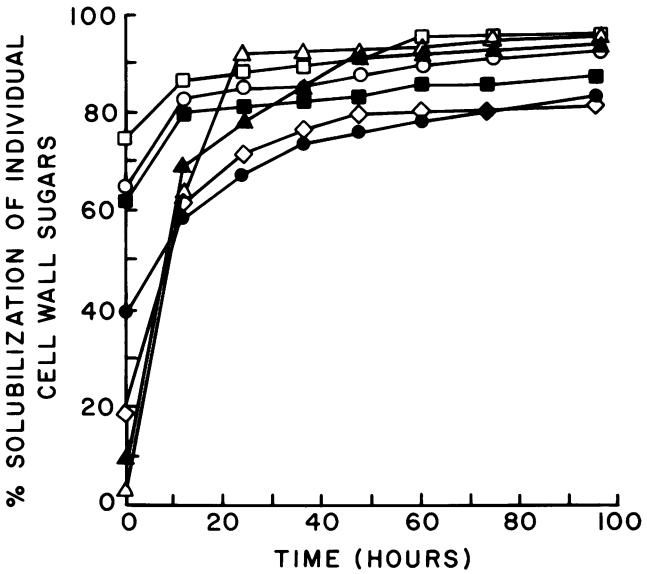

FIG. 5. Solubilization of carbohydrate from ACW by *R. albus* 8 during growth. Cultures (12 ml) containing 18 mg of ACW were grown for 96 h as described in the text. Zero-time data points represent the percentage of each residue in the soluble fraction after autoclaving but before inoculation. Subsequent data indicate the course of the removal of each sugar from the insoluble fraction of ACW during the culture period. Procedures for data calculation are described in the text. Symbols: △, glucose; ▲, xylose; ◇, mannose; ●, galactose; ■, rhamnose; □, arabinose; ○, uronic acids.

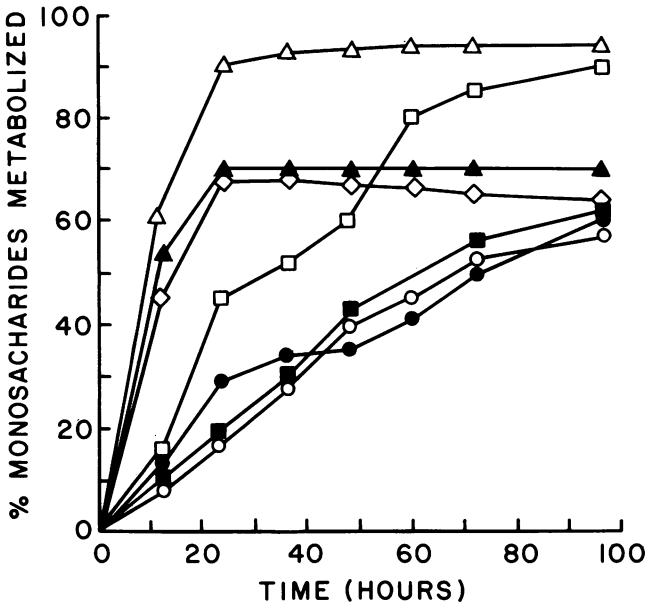

FIG. 6. Metabolism of carbohydrate by *R. albus* 8 during growth on ACW. Cultures (12 ml) containing 18 mg of ACW were grown for 96 h as described in the text. The quantities of each monosaccharide available (the sum of the soluble and insoluble fractions) in the zero-time control (Table 1) represent the 100% value for each sugar. Assuming 0% of each sugar has been metabolized at zero time, subsequent data points are the total (on a percentage basis) of each monosaccharide that had been removed from the culture at each time point (i.e., the zero-time amount less the sum of that measured in the soluble and insoluble fractions). Symbols: △, glucose; ▲, xylose; ◇, mannose; ●, galactose; ■, rhamnose; □, arabinose; ○, uronic acids.

R. albus 8 utilizes both glucose and xylose in preference to the other sugars in ACW (Table 3) even though all of the ACW sugar components are rendered soluble at approximately the same rate (Fig. 5). As Dehority (5) has postulated, the solubilization of these other carbohydrates by *R. albus* 8 may be necessary to obtain the maximum amounts of glucose and xylose from ACW. It is interesting to note that those sugars which are utilized more slowly (arabinose, galactose, rhamnose, and uronic acids) are often associated with wall peptic polymers. The data of Morris and van Gylswyck (12) also indicate a delayed utilization of uronic acids by a variety of rumen bacteria, including *R. albus*, although because their substrate was derived from a monocot, the uronsyl residues could have been associated with hemicellulose. The pectins are more soluble than the hemicelluloses and cellulose which contain the bulk of the glucose and xylose in dicot cell walls (11). Dehority (15) has demonstrated that non-cellulolytic rumen bacteria use the carbohydrates solubilized but not utilized to a great extent by the cellulolytics. The data reported here clearly show that *R. albus* will use pectic substrates when preferred carbon sources become limiting.

TABLE 3. Time course of utilization of individual ACW sugars by *R. albus* 8[a]

Sugar	Amt (mg) utilized at given culture age		
	24 h	48 h	96 h
Glucose	6.0	6.8	6.8
Xylose	0.9	1.0	1.0
Mannose	0.1	0.1	0.1
Arabinose	0.5	0.7	0.9
Galactose	0.2	0.2	0.3
Rhamnose	0.1	0.1	0.2
Uronic acid	0.6	1.5	1.9
Total	8.4	10.3	11.2
Total used in previous 24 h	8.4	1.9	0.9

[a] The amount of each sugar in the 18 mg of ACW supplied to cultures is given in Table 1. The glucose includes both cellulosic and noncellulosic fractions.

ACKNOWLEDGMENT

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