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Effect of Lytic Enzymes of *Acanthamoeba castellanii* on Bacterial Cell Walls

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Extracts of *Acanthamoeba castellanii* (Neff) contain α- and β-glucosidase, β-galactosidase, β-*N*-acetylglucosaminidase, amylase, and peptidase. All of these activities are optimal between *p*H 3 and 4. These extracts also were found to clarify suspensions of cell walls from nine different gram-positive bacteria, including *Micrococcus lysodeikticus*. The *p*H optimum for the lytic activity was between 3 and 4. The extent of lysis of the various cell walls did not correlate with the release of free amino groups and of free *N*-acetylated sugars from the walls during digestion with these extracts. Suspensions of cell walls of *Escherichia coli* (a gram-negative bacterium), *Cordiceps militaris* (a fungus), and *Acanthamoeba* cysts, as well as of colloidal chitin, were not clarified by incubation with these extracts, although reducing sugars were released from each of these materials. Exhaustive digestion of *M. lysodeikticus* walls by lysozyme released no free *N*-acetylglucosamine. The products of exhaustive digestion of this cell wall with *Acanthamoeba* extracts were free *N*-acetylglucosamine, free *N*-acetylmuramic acid, glycine, alanine, glutamic acid, lysine, and *N*-acetylmuramic acid peptide fragments. These results suggest that the amoeba extracts contain endo- and exo-hexosaminidases, in addition to β-hexosaminidase and peptide hydrolases.

An increasing interest in glycosidases, peptidases, and proteolytic enzymes for use in structural determination has been stimulated by recent studies of cell wall structure in a variety of microorganisms. One of the enzymes used most frequently for studies of bacterial cell walls has been egg white lysozyme, endo-*N*-acetylmuramidase (13, 33). However, the cell walls of many bacteria are resistant to the lytic activity of lysozyme, either because lysozyme does not act on peptidoglycan, which contains muramic acid residues with *O*-acetyl groups on C_6 (3), or because the peptidoglycans are masked by other lysozyme-insensitive elements. A number of lytic enzymes which degrade peptidoglycan to complementary β-*N*-acetylmuramyl-*N*-acetylglucosamine disaccharides, either alone or in combination with peptide fragments, have been isolated from bacterial sources (2, 4, 11, 30). Other lytic enzymes have been isolated from *Streptomyces* (5) and from fungi (9). Recently, Malchow et al. (14) indicated that cellular slime molds also contain enzymes that digest lipolysaccharide of gram-negative organisms in vivo. In this paper, we have investigated the lytic activity of enzymes from *Acanthamoeba castellanii* on a variety of cell walls.

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MATERIALS AND METHODS

Culture techniques and preparation of crude extracts. *Acanthamoeba* sp. (16) was maintained and grown at room temperature as described by Weisman and Korn (34). Recently, this organism has been found to be identical with *A. castellanii* (19). For preparation of small batches of enzyme extracts, cells were taken from 1-liter shaking cultures growing in a 3-liter low culture flask. Alternatively, larger amounts of cells were obtained from 16-liter cultures grown in 20-liter carboys with aeration at the rate of about 6.5 ft^3 (1,820 liters) per hr. In either case, cells were grown for about 5 or 6 days and were harvested before the total cyst count of the preparation exceeded 3%. All enzyme extractions and fractionation procedures were carried out at 4 C. The cells were collected at 400 to 600 × *g* and washed in 0.05M sodium acetate buffer (*p*H 5.0), suspended in a suitable volume of this buffer, and immediately broken by passage through a French press. The extract was then subjected to centrifugation at 30,000 × *g* for 30 min. The supernatant extract so obtained was used as a source of enzymes and is referred to as the crude extract. The crude amoeba extracts were turbid and contained considerable amounts of endogenous polysaccharides. These endogenous polysac-

charides were removed by either of the following procedures: the crude extract was dialyzed against 0.05 M sodium acetate buffer (*p*H 5.0) overnight at 4 C and then centrifuged at 37,000 × *g* for 30 min to obtain a clear supernatant liquid, or the crude extract was fractionated with solid ammonium sulfate to 55% saturation to obtain a clear supernatant solution which had a reduced total enzyme activity. Both of these procedures yielded enzyme fractions which did not release reducing sugars in the absence of a polysaccharide substrate. All extracts were stored at −20 C.

Preparation of cell walls. Cell walls of the filamentous form of the fungus *Cordiceps militaris* were the generous gift of A. J. Guarino (A. Guarino, D. Marks, and B. Keller, *unpublished data*). Bacterial cell walls were prepared by the method of Sharon and Jeanloz (27) from *Micrococcus lysodeikticus* (obtained from Miles Laboratories, Inc., Elkhart, Ind.) and from *Escherichia coli* B; these organisms were grown on Frazier's medium and harvested during exponential growth. Cell walls of *Bacillus cereus*, log-phase *Streptococcus faecalis* (treated with sodium dodecyl sulfate to destroy autolytic activity), stationary-phase *S. faecalis* (valine deprived), *Sarcina lutea*, *Bacillus subtilis*, *B. megaterium* KM, *M. roseus*, and *M. radiodurans* were the generous gift of G. D. Shockman.

Encystment was induced in a culture of *Acanthamoeba* as described by Neff et al. (18) in a medium containing, in grams per liter: KCl, 7.9; 2-amino-2-methyl-1,3-propandiol, 2.1; $MgSO_4 \cdot 7H_2O$, 2.1; and $CaCl_2 \cdot 2H_2O$, 0.6. The *p*H was adjusted to 7.0 with HCl. Amoebae were allowed to encyst for 36 hr at room temperature, and the cyst walls were prepared by slight modification of the method of Tomlinson and Jones (31). The cysts were collected by centrifugation at 600 × *g* at 2 C, washed, and suspended in 0.05 M sodium acetate buffer (*p*H 4). The cells in the suspension were then homogenized in a Potter-Elvehjem type homogenizer to rupture any remaining amoebae. Microscopic examination of the homogenate indicated that over 98% of the remaining intact cells were mature cysts. After being washed in acetate buffer, the cysts were broken by three passes through a French press, and the resulting suspension was subjected to centrifugation at 30,000 × *g* for 30 min in the cold. The pellet was suspended in deionized distilled water with homogenization by hand, and the cyst walls were isolated by alternately centrifuging the suspension for 5 min at 600 × *g* at 2 C and washing it with deionized distilled water until the supernatant solutions were clear. The walls were then frozen and lyophilized.

Colloidal chitin was prepared according to the method of Reynolds (25).

Analytical procedures. Reducing groups were determined by the ferricyanide procedure (20). *N*-acetylated sugars were measured by the procedure of Reissig et al. (24), with the use of a heating time of 30 min. The amount of *N*-acetylated sugars was calculated with *N*-acetylglucosamine as a standard. Neutral sugars were determined by the anthrone method of Morris (15). Free amino groups were determined by the fluorodinitrobenzene technique of Ghuysen et al. (8), on a 5- to 10-fold larger scale.

Enzyme assays. Glycosidase activity was assayed by incubation with the appropriate synthetic *p*-nitrophenyl substrate (Calbiochem, Los Angeles, Calif.). Incubations were terminated by diluting the reaction mixtures 20-fold or more with 0.1 M Na_2CO_3. The dilution was then clarified by centrifugation, and the absorbance of the supernatant solution was measured at 420 nm. The data reported for reaction mixtures represent values from which the values of control reaction mixtures run without substrate and, separately, without enzyme extract have been subtracted. The molar extinction coefficient of *p*-nitrophenol in 0.1 M Na_2CO_3 at 420 nm was calculated to be 1.3×10^7 cm^2 $mole^{-1}$ from a standard solution of *p*-nitrophenol. Even with reaction mixtures of the lowest *p*H values used in these experiments, the *p*H of the dilution in Na_2CO_3 was not changed enough to affect this value.

Column chromatography. Gel filtration was carried out with Bio-Gel P-10, 100 to 200 mesh (Calbiochem), equilibrated with 0.1 M sodium chloride. The columns were calibrated with blue dextran and *N*-acetylglucosamine. Preliminary experiments in which distilled water was used for developing the columns gave poor elution patterns which did not reflect the molecular size of the products.

Paper chromatography and electrophoresis. Paper chromatography was carried out by the descending technique on Whatman 3 MM paper with the use of *n*-butyl alcohol-acetic acid-water (63:10:27)or pentanol-pyridine-water (7:7:6). Paper electrophoresis was carried out on Whatman 3MM paper in a High Voltage Electrophorator model D (Gilson Medical Electronics, Middleton, Wis.), with the use of acetic acid-formic acid buffer, *p*H 1.85 (78 g of glacial acetic acid and 26.6 g of 88% formic acid per liter). Reducing sugars were detected with silver nitrate reagent (26). Amino acids and amino sugars were detected with ninhydrin reagent (1% ninhydrin in acetone buffered with 0.05 M phosphate at *p*H 6.8).

RESULTS

Enzymatic activities in crude extracts. Crude extracts of *Acanthamoeba* had a variety of enzymatic activities (Table 1). β-Glucosidase, α-glucosidase, β-galactosidase, β-*N*-acetylglucosaminidase, and protease activities were observed. All of these activities were optimal between *p*H 3 and 4 (Fig. 1). With glycogen as substrate, particularly at the optimal *p*H, amylase activity was observed with the crude extracts in the absence of added substrate. This activity, which increased with incubation time, presumably represents autodigestion of endogenous polysaccharides present in the extracts. Endogenous polysaccharides were removed from the crude extracts by the procedures described in Materials and Methods to obtain the extracts which were used in all experiments except those reported in Table 1.

TABLE 1. *Hydrolytic activity of crude extracts*

Expt	Substrate	Specific activity
I[a]	*p*-Nitrophenyl-α-D-glucopyranoside	31.1[b]
	p-Nitrophenyl-β-D-glucopyranoside	65.1
	p-Nitrophenyl-β-D-galactopyranoside	14.0
	p-Nitrophenyl-*N*-acetyl-β-D-glucosamide	300.6
II[c]	Glycogen	0.013[d]
III[e]	Hemoglobin	0.006[f]

[a] The reaction mixture contained substrate (1 mM) as indicated and 0.11 mg of protein from an amoeba extract in a total volume of 1.0 ml of 0.01 M sodium acetate buffer (*p*H 4). Incubation was for 10 min at 30 C. Afterwards, the reaction mixture was diluted 20-fold with 0.1 M sodium carbonate, and the absorbance at 420 nm was measured. *p*-Nitrophenol release was then calculated as described in Materials and Methods.

[b] Micromoles of *p*-nitrophenol released per milligram per minute.

[c] The reaction mixture contained 0.3% rabbit liver glycogen and 1.95 mg of protein from an amoeba extract in a total volume of 0.3 ml of 0.03 M tris(hydroxymethyl)aminomethane-citrate buffer, *p*H 3.5. Incubation was for 60 min at 30 C. Reducing power release was measured (28) with glucose as a standard. Values from reaction mixtures containing only extract and only glycogen have been substracted.

[d] Micrograms of reducing sugar per milligram per hour.

[e] The reaction mixture of 1.0 ml contained 1.2 mg of amoeba extract protein, 0.25 ml of hemoglobin solution (8%, w/v), and 0.2 M sodium acetate buffer (*p*H 4.0). Incubation was at 45 C for 1 hr. The amount of protein solubilized was measured at 280 nm after treatment with 5 ml of 3% trichloroacetic acid, as described by Barrett (1). Control values of samples in the absence of hemoglobin were subtracted from the values of the complete incubation.

[f] Change in absorbancy at 280 nm per milligram per minute.

Degradation of various cell walls by amoeba extracts. Because of the relatively high specific activity of the β-*N*-acetylglucosaminidase (Table 1), the extracts were tested for their lytic activity on *M. lysodeikticus* cell walls and on cell walls from a variety of other gram-positive organisms (Fig. 2). In addition to lytic activity, the release of compounds containing free amino groups and *N*-acetylated sugars from the cell wall was measured (Table 2). From Fig. 2 and Table 2, it is obvious that there is lysis of a broad spectrum of gram-positive cell walls as well as a wide variation in the amounts of free amino groups and *N*-acetylated sugars released during the lytic process. Cell walls from a fungus (*C. militaris*), colloidal chitin from lobster shells, cell walls

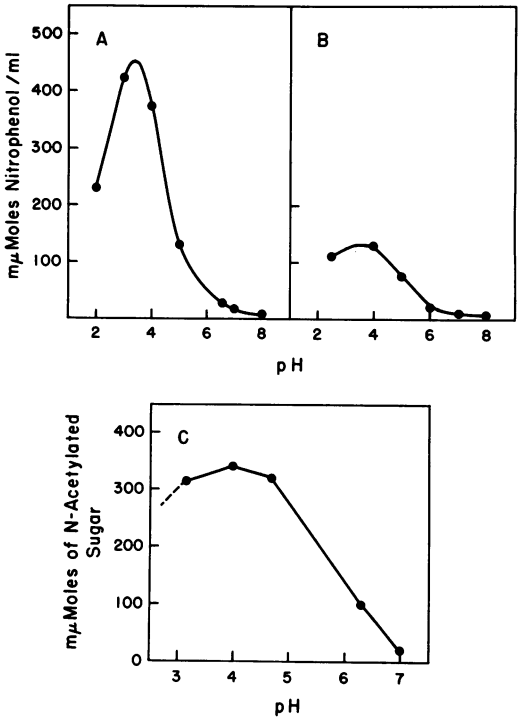

FIG. 1. *Optimal pH for the hydrolysis of nitrophenylglucosides and of M. lysodeikticus cell walls by the amoeba extract. (A) Reaction mixtures contained 4.5 mmoles of α-nitrophenylglucoside and 0.05 mg of protein from an amoeba extract in a total volume of 0.3 ml of a suitable combination of citric acid and tris(hydroxymethyl)aminomethane (Tris), giving the indicated pH at a final concentration of 0.033 M. (No significant differences were observed when Na_2CO_3 replaced Tris in these buffers.) Incubation was for 60 min at 30 C. Postincubation treatment was as described in Materials and Methods. The specific activities of the α-glucosidase activity vary from experiment to experiment owing to its instability (Rosenthal and Hardman, unpublished observations). (B) The reaction mixtures and conditions were the same as for A except that β-nitrophenylglucoside was the substrate and 0.2 mg of protein from a different amoeba extract was used. (C) Reaction mixtures contained 1 mg of M. lysodeikticus cell walls and 0.42 mg of protein from an amoeba extract in a total volume of 0.8 ml of a suitable combination of citric acid and potassium phosphate or sodium acetate and acetic acid to give the indicated pH at a final concentration of 0.05 M. Incubation was for 60 min at 37 C, after which the reaction was stopped by heating in a boiling-water bath for 5 min. Samples were then taken for assay of N-acetylated sugars as described in Materials and Methods. Values reported are for 0.08 ml of incubation mixture.*

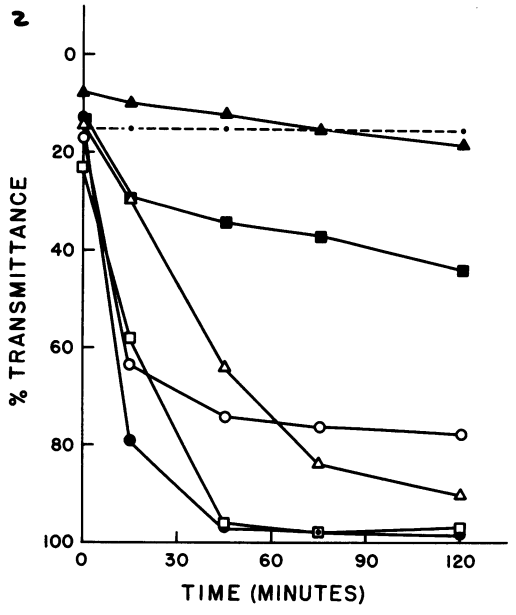

FIG. 2. *Lysis of cell walls from gram-positive bacteria during digestion with amoeba enzymes. Reaction mixtures were set up as described in Table 2. The per cent transmittance at 650 nm was recorded on a Bausch & Lomb Spectronic-20 colorimeter at the times indicated. Dashed line, undigested M. lysodeikticus cell wall suspension;* ▲, *B. cereus;* △, *M. roseus;* ■, *B. subtilis;* □, *S. faecalis (sodium dodecyl sulfate);* ●, *M. lysodeikticus;* ○, *S. lutea.*

from a gram-negative bacterium (*E. coli*), and the cyst form of *Acanthamoeba* were also tested (Table 3).

Comparison of M. lysodeikticus cell wall digestion products with lysozyme and amoeba enzymes. In view of the broad spectrum of glucosidase activity in amoeba extracts (Table 1) and the action of these extracts on a variety of cell walls (Table 2), the effects of these enzymes on *M. lysodeikticus* cell walls were examined in more detail. It was of interest to see whether the amoeba enzymes would be useful in studying cell wall structure, and in particular whether enzymatic activities other than lysozyme were involved in the lytic activity observed.

In preliminary experiments, 18-hr digestion of cell walls with the amoeba enzymes or with lysozyme produced complete solubilization of the cell walls. The 55% ammonium sulfate supernatant fraction from the extracts release about seven times as much *N*-acetylated sugar as did lysozyme after 18 hr. The products of separate large-scale incubations of cell walls with lysozyme and with amoeba enzymes were examined by gel filtration. The lysozyme incubation, which

TABLE 2. *Release of free amino groups and N-acetylated sugars from the cell walls of gram-positive bacteria during digestion with amoeba enzymes*[a]

Cell wall	Free NH_2 groups released	*N*-acetylated sugars released	Clarification[b]
	nmoles/mg	*nmoles/mg*	%
M. lysodeikticus	73	410	98
S. faecalis (sodium dodecyl sulfate)	0	730	96
M. roseus R 27	0	380	89
B. megaterium	75	880	86
S. lutea	161	150	72
S. faecalis (valine)	22	760	65
M. radiodurans	26	600	59
B. subtilis	65	510	32
B. cereus	157	70	11

[a] Cell walls were incubated at 37 C in a total volume of 4.0 ml of 0.05 M sodium acetate buffer (*p*H 4.0). Cell wall concentration was 1.5 mg of wall per ml of incubation mixture, except in the cases of *S. faecalis* (1.4 mg/ml), *B. megaterium* (1.08 mg/ml), *M. roseus* (1.6 mg/ml), and *M. radiodurans* (0.73 mg/ml). Enzyme concentration in all cases was 0.25 mg of protein per ml. Samples taken after 2 hr of incubation were boiled to inactivate the enzymes present and then assayed as described in Materials and Methods. Clarification of the cell wall suspensions was measured as a decrease in turbidity at 650 nm according to the equation given.

[b] Per cent clarification = $(\% T - \% T_0)/(100 - \% T_0) \times 100$, where $\% T$ is measured at the end of the incubation and $\% T_0$ is measured at zero time.

TABLE 3. *Effect of amoeba extracts on various cell walls*[a]

Source of cell wall	Lysis	Reducing power released[b]
M. lysodeikticus	+	1,630
E. coli	−	135
Cordiceps militaris	−	110
Acanthamoeba cysts	−	10
Colloidal chitin	−	6

[a] Digestion was carried out at *p*H 3.9 in 0.5 M sodium acetate buffer for 18 hr at 37 C in a reaction vessel containing 15 mg of cell wall per ml and 88 μg of amoeba protein which had been dialyzed overnight against sodium acetate buffer at 4 C.

[b] Nanomoles per milligram of cell wall.

contained 140 μg of egg white lysozyme (Mann Research Laboratories, New York, N.Y.) and 50 mg of cell walls in 2.0 ml of 0.05 M ammonium acetate (*p*H 7.4), was incubated at 37 C for 18 hr with a layer of toluene. At the end of the in-

cubation period, the solution was applied to a Bio-Gel P-10 column. The elution pattern of this column is shown in Fig. 3A. The amoeba enzyme digestion was carried out with 50 mg of cell walls and 0.2 ml of 55% supernatant fraction from a dialyzed extract in 1.05 ml of 0.05 M sodium acetate buffer (*p*H 4.0) for 18 hr at 37 C with a layer of toluene. At the end of the incubation period, the solution was heated at 100 C for 10 min, and then a sample was applied to the Bio-Gel P-10 column. The elution pattern is shown in Fig. 3B. With lysozyme (Fig. 3A), the glucose-aminomannuronic acid polymer (21; S. Rosenthal and E. J. Reed, Federation Proc. 27:834, 1968) and large fragments of peptidoglycan were excluded from the gel (30 to 50 ml), whereas smaller fragments eluted between 50 and 100 ml. The low values for *N*-acetylated sugars result from the fact that the color yield for oligosaccharides is very low in the Morgan-Elson assay as compared with the reducing sugar assay (12). Compared with the lysozyme digest, one striking difference in the elution pattern of the amoeba enzyme digest is the presence of significant amounts of material eluting between 100 and 115 ml. This suggests that the amoeba enzymes but not lysozyme result in the release of monosaccharides, since *N*-acetylglucosamine was found to elute in this volume on calibration of the Bio-Gel column. Furthermore, in this region the analyses for *N*-acetylated sugars and reducing sugars are in good agreement, as would be expected if the material eluting in this region were made up of monosaccharides. The amoeba enzymes did not appear to affect the glucose-aminomannuronic acid polymer, as indicated by the exclusion of all the neutral sugar from the Bio-Gel P-10.

Identification of the products of M. lysodeikticus cell wall digestion with amoeba enzymes. The dialyzed amoeba enzyme fraction free from endogenous polysaccharides contained a considerable amount of protease activity which caused a marked loss of enzyme activity during prolonged digestion periods. To obtain maximal release of cell wall digestion products, incubations were carried out either in the presence of larger amounts of enzyme or with several additions of enzyme. The amount of reducing groups and *N*-acetylated sugars released from the walls was determined after the incubation. Based on *N*-acetylglucosamine as standard, the release of 1.1 to 1.2 μmoles of *N*-acetylated sugar per mg of cell wall was observed. Using acid hydrolysis, Katz and Strominger (10) found that these walls contain a total of 0.96 μmole of glucosamine plus muramic acid per mg of cell walls.

The products of exhaustive cell wall digestion

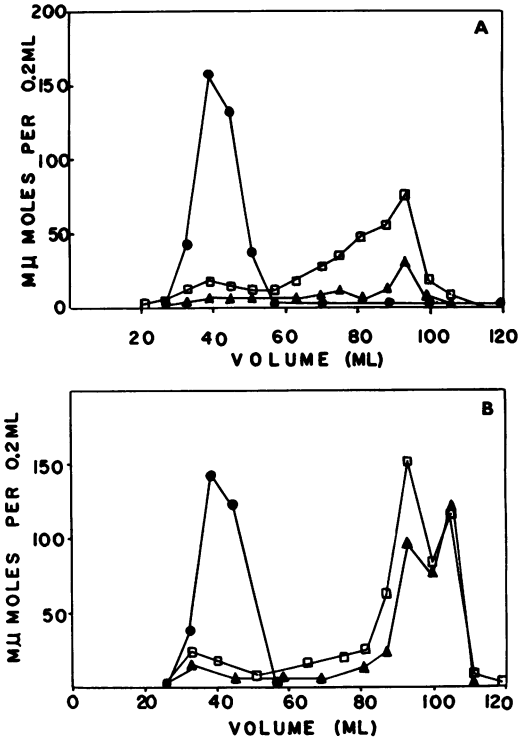

FIG. 3. *Separation of the products of lysozyme or amoeba enzyme digestion of M. lysodeikticus cell walls on Bio-Gel P-10 with 0.1 M sodium chloride. The Bio-Gel P-10 column used was 1.5 × 100 cm. The chromatography was carried out at room temperature. The elution volume of dextran blue was 29 ml and that of N-acetylglucosamine was 105 ml. Analyses of material eluted from the column are based on values calculated with the standards in parentheses: ●, neutral sugar (glucose); □, reducing sugar (N-acetylglucosamine); and ▲, N-acetylated sugar (N-acetylglucosamine). A: 22.5 mg of cell walls digested with lysozyme was applied to the column in 0.9 ml; 3-ml fractions were collected and assayed as described in Materials and Methods. B: 21.4 mg of cell wall digested with the 55% ammonium sulfate supernatant fraction from a dialyzed amoeba extract was applied to the column in 0.45 ml; 3-ml fractions were collected and assayed as described in Materials and Methods.*

were examined by paper chromatography. Free *N*-acetylglucosamine and *N*-acetylmuramic acid appeared in significant quantities after exhaustive digestion with amoeba enzymes (Fig. 4). The identity of these products was confirmed by electrophoresis at *p*H 1.85 after deacetylation of the sugars obtained from preparative paper chromatography.

A third and as yet unidentified compound with a lower R_F than that of *N*-acetylglucosamine was also observed. Paper electrophoresis at *p*H 1.85

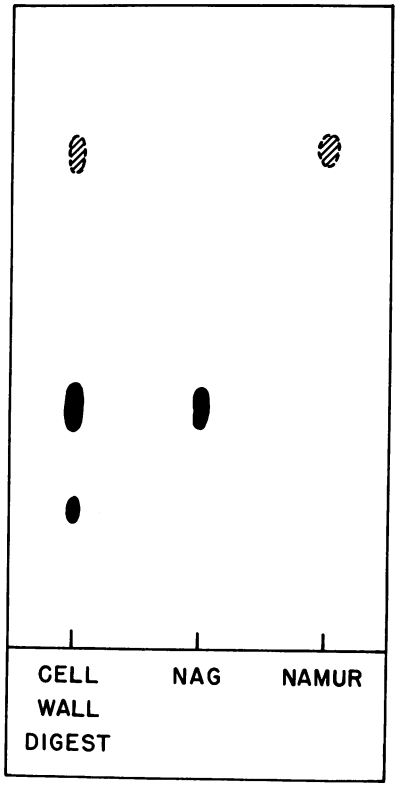

FIG. 4. *Paper chromatography of the products of digestion of M. lysodeikticus cell walls with amoeba extracts. M. lysodeikticus cell walls (15 mg) were incubated at 37 C for 48 hr with 245 μg of protein from an amoeba extract in 1 ml of 0.05 M sodium acetate buffer (pH 4.0). Two drops of toluene were added. After incubation, the reaction mixture was boiled for 5 min. A 25-μliter sample was chromatographed in n-butyl alcohol-acetic acid-water (63:10:27), and reducing sugars were detected with silver nitrate reagent as described in Materials and Methods. NAG = N-acetylglucosamine; NAMUR = N-acetylmuramic acid.*

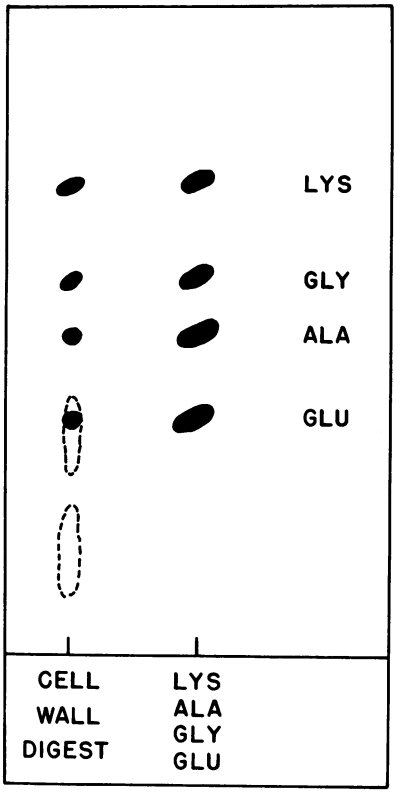

FIG. 5. *Paper electrophoresis of the products of digestion of M. lysodeikticus cell walls with amoeba extracts. The cell wall digest was the same as described in Fig. 4. Electrophoresis was carried out on a 25-μliter sample as described in Materials and Methods. Free amino groups were detected with ninhydrin stain. Dotted spots refer to material which stained less intensely. LYS = lysine; ALA = alanine; GLY = glycine; GLU = glutamic acid.*

was used to identify the products containing free amino groups which were released during this digestion. The four cell wall amino acids (alanine, glycine, glutamic acid and lysine), along with significant amounts of peptide material, were found (Fig. 5). At the dilution used for electrophoresis, amino acids released by autodigestion of the amoeba proteins were not visible.

DISCUSSION

Extracts of *A. castellanii* (Neff) contain a variety of glucosidase activities. Among these are α- and β-glucosidases, β-galactosidase, and β-*N*-acetylglucosaminidase. The extracts also mediate an enzymatic lysis of cell walls from several species of gram-positive bacteria. *Acanthamoeba* can utilize maltose, cellobiose, sucrose, or lactose as a carbon source (17). The glycosidases examined here may reflect at least some of the enzymes involved in the utilization of these disaccharides. A determination of the extent to which other enzymes are involved must await the purification and separation of the glycosidase activities along with the testing of *p*H optima and substrate specificity.

Acanthamoeba ingests particulate material by phagocytosis (35) and can utilize several species of bacterial cells as foodstuffs (16). Lysosomes have been thought responsible for the digestion of engulfed materials by contributing their hydrolytic enzymes to the phagocytic vacuole

(J. G. Hirsch and Z. A. Cohn, Federation Proc. 23:1023, 1964). The various glycosidases, proteases, and lytic enzymes of the amoeba extract so far studied may be of the lysosomal type in view of their occurrence together and their *p*H optimum. It seems likely that the broad spectrum of gram-positive bacterial walls lysed by these extracts reflects the ability of this soil amoeba to devour bacterial cells. Upadhyay (32) found that extracts of a related amoeba, *Hartmannella glebae*, caused the lysis of a variety of gram-positive but not gram-negative bacterial cells.

The amoeboid form of *Acanthamoeba* undergoes encystment during conditions of starvation. *Acanthamoeba* extracts, prepared from the amoeboid form, did not cause significant lysis of isolated cyst walls under the conditions employed. Excystment of a related amoeba, *H. astronyxis*, occurs without a widespread digestion of the cyst wall. The trophozoite emerges through an aperture caused by the dislodging of an operculum, leaving the empty cyst case behind (23).

Recent detailed studies of cell wall structure in gram-positive organisms have been stimulated by the isolation of a variety of enzymes which hydrolyze either peptide or *N*-acetylhexosamine linkages. Exhaustive digestion of *M. lysodeikticus* cell walls with *Acanthamoeba* extracts releases free *N*-acetylglucosamine, *N*-acetylmuramic acid, glycine, alanine, glutamic acid, lysine, and *N*-acetylmuramic acid peptides. This suggests that the extracts contain both endo- and exo-hexosaminidases, β-*N*-acetylhexosaminidases and peptide hydrolases.

The cell wall of gram-positive organisms contains peptidoglycan and at least one other polysaccharide component. Although only minor variations in the peptidoglycan composition can be found, a wide variety of different types of cross-linkages occurs. These variations partially account for the differences in sensitivity to lysis of cell walls by bacteriolytic enzymes. *O*-acetylation of the sugar moieties (7) and the presence of polysaccharides other than peptidoglycan are known to make some cell walls resistant to enzymatic hydrolysis (6).

A. castellanii contains a broad spectrum of enzymes which act on both the glycan and peptide portion of the peptidoglycan. Examination of the data obtained for *N*-acetylated sugars after exhaustive digestion of *M. lysodeikticus* walls indicates that both endo-hexosaminidases and β-*N*-acetylhexosaminidases are present, since essentially all of the *N*-acetylated sugars in the peptidoglycan are released as monosaccharides. A number of peptide hydrolases are presumably also present, since all four of the cell wall amino acids appear after 48 hr of enzymatic digestion. On fractionation of the amoeba extracts, two lytic enzymes and a β-*N*-acetylhexosaminidase have been obtained (S. Rosenthal, D. Hardman, and L. Klunk, *unpublished data*). Investigation of the peptidase enzymes has not been pursued.

Large differences in susceptibility to lysis of various gram-positive cell walls digested with amoeba enzymes are apparent from the data in Table 2. However, no correlation between the lysis and the release of either free amino groups or *N*-acetylated sugars is apparent. Compare, for example, the data for *M. lysodeikticus* with data for *M. roseus* or *M. radiodurans*.

The appearance of all four cell wall amino acids during electrophoresis of *M. lysodeikticus* walls after enzymatic digestion clearly indicates that peptide hydrolases are present. However, with *S. faecalis* and *M. roseus* walls, where peptide cross-linkages involve threonine (22) and aspartic acid moieties (6) not found in most mucopeptides, no free amino groups are released during the digestion which results in cell wall lysis. This suggests that the peptidase activity is dependent on the arrangement and cross-linkages of the peptide side chains.

In other cell walls, such as those of *B. cereus* and *S. lutea*, there is extensive release of free amino groups and very little, if any, *N*-acetylated sugar release. However, *B. cereus* walls resist lysis, whereas *S. lutea* walls are rapidly lysed. Although it is not possible to account for these differences at present in terms of the structure of these walls, the presence of peptidases with substrate specificities other than those reported in the literature seems likely.

Cell walls other than the walls of the gram-positive organisms tested here were not lysed. This includes cell walls from *E. coli*, *C. militaris* (a fungus), and *Acanthamoeba* cysts, and chitin isolated from lobster shells. Although lysis was not observed, there was evidence for the release of reducing sugars from these cell walls. Weinbaum (*personal communication*) has found that the pellet from a peptidoglycan fraction of *E. coli* first treated with lysozyme was partially solubilized with additional release of *N*-acetylated sugars when incubated further with these *Acanthamoeba* extracts.

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