Low Molecular Weight Substrates for Lysozyme

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A Thesis Describing Chemical Research in Ch 80

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Preparation of short chain polymers of N-acetylglucosamine by acid hydrolysis of chitin was undertaken. Separation of the various chain lengths of the hydrolysis breakdown products was done by column chromatography on charcoal-celite with an ethanol solvent.

Preparation of the dimer and tetramer of N-acetylglucosaine-N-acetylmuramic acid was undertaken by lysozyme digestion of the purified cell walls of Micrococcus lysodeikticus. Separation was again accomplished by chromatography.

At the time when this work was performed (1965) lysozyme was a particularly well characterized enzyme. A great deal of work had been performed to discover the amino acid sequence, the crystal structure and its various physical properties, yet relatively little was known about its enzymatic activity.

Studies of lysozyme catalyzed degradation of chitin and of bacterial cell walls have indicated the general nature of the compounds that are hydrolyzed by lysozyme. Berger and Weiser[1] found that lysozyme degrades chitin, which is a $\beta(1\rightarrow4)$N-acetyl-glucosamine polymer. Apparently lysozyme belongs to the N-acetylhexosaminidase group of enzymes. Meyer, Palmer, Thompson and Khorazo[2] had found that lysozyme did not attack α-glycosidic bonds. This, coupled with Berger and Weiser's work makes it seem that lysozyme is specific for β-glycosidic bonds.

It was felt that what was needed at this point were experiments upon small saccharide substances of known structure in order to determine the specificity and kinetic properties of

lysozyme. In order to prepare such substrates two general methods of procedure were undertaken: 1. The acid hydrolysis of chitin 2. Lysozyme digestion of the cell walls of Micrococcus lysodeikticus. In both cases a long chain polymer is apparently broken down into smaller components. For studies of specificity and kinetics it is necessary to separate these breakdown components into distinct entities. This was accomplished by chromatography.

Acid Hydrolysis of Chitin - Experimental

Hydrolysis of chitin was accomplished by the method of Rupley:[3] 20g of powdered chitin were dissolved in 350ml of cold concentrated hydrochloric acid kept at 0°C. The dissolved chitin was then incubated for two hours at 40° to hydrolyze it. It was then neutralized with a saturated sodium hydroxide solution, keeping the temperature below 5°, and then filtered. The resulting solution was then partially evaporated.

In order to separate the hydrolysis components the solution was chromatographed on a charcoal-celite column with a linear gradient from 0 to 60% ethanol. The column was prepared as follows: Celite was washed overnight with concentrated hydrochloric acid, washed free of the acid and dried in an oven. 50g of Darco G-60 carbon and 50g of the acid washed celite were mixed for thirty minutes with 400ml of 2.5% stearic acid in absolute ethanol. The mixture was filtered and then remixed with 400 ml of 50% ethanol saturated with stearic acid. The mixture

was then refiltered and suspended in 10% ethanol. This slurry was poured into the column.

Nelson's colorometric modification of the Somogyi micro-copper method was used to test for the presence of reducing sugars in the chromatographic fractions.[4] The amount of reduced copper is determined by reaction with arsenomolybdate color forming reagents:

A. Low alkaline copper reagent: 12g of Rochelle's salt and 24g of anhydrous sodium carbonate are dissolved in 250 ml of water. A solution of 4.0g of cupric sulfate pentahydrate is added with stirring, followed by 16g of sodium hydrogen carbonate. A solution of 180g of anhydrous sodium sulfate in 500 ml of water is boiled to expel air, then the two solutions are combined and diluted to one liter. After standing for one week the clear supernatant is used.

B. Arsenomolybdate reagent: to 25g of ammonium molybdate in 450ml of water, 21 ml of 96% sulfuric acid is added followed by sodium hydrogen arsenate heptahydrate dissolved in 25 ml water. The mixed solution is incubated at 37° for twenty four hours and stored in a glass stoppered bottle in the dark.

The test procedure is: To 1-5 ml. of sugar solution containing not more than 0.6mg of D-glucose or its equivalent, an equal volume of the low alkalinity copper reagent is added. Samples blanks and standards are heated for ten minutes in a vigorously boiling water bath and then cooled. One ml of the arsenomolybdate reagent is added to determine 0.1mg or less of D-glucose and 2ml are added for 0.1 to 0.6 mg of D-glucose.

After all the cuprous oxide is dissolved, the solution is diluted to an appropriate volume and allowed to stand at least fifteen but no longer than forty minutes. Absorbances are read at 500 mu.

A further test of N-acetylamino sugars was performed utilizing spray reagents upon samples spotted on chromatographic paper. The method used was that of Salton:[5] Use of an ethanol-borate spray followed by steaming and spraying with a dimethyl-aminobenzaldehyde spray. The reagents are as follows:

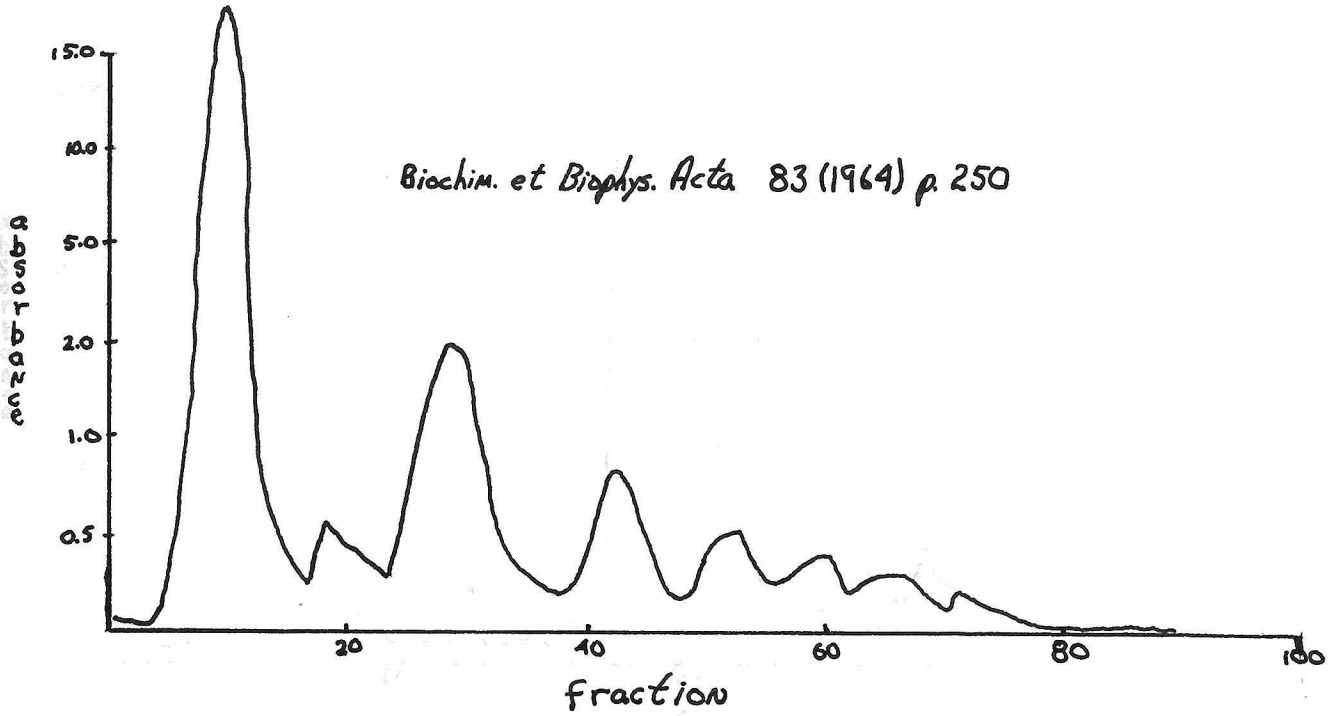
Solution A: 100 ml of 95% ethanol and 100ml of 0.05 molar $Na_2B_4O_7$.

Solution B: 20ml of 2% solution of p-dimethylaminobenzal-dehyde in glacial acetic acid, 60ml of n-butanol and 0.8ml of concentrated hydrochloric acid.

The paper with the sample is sprayed with solution A, suspended in steam for ten minutes and then sprayed with solution B. Appearance of a purple spot indicates the presence of N-acetyamino sugars.

Results:

The hydrolysis procedure was performed several times and lead each time to rather ambiguous results. Rupley reported results of the following form:



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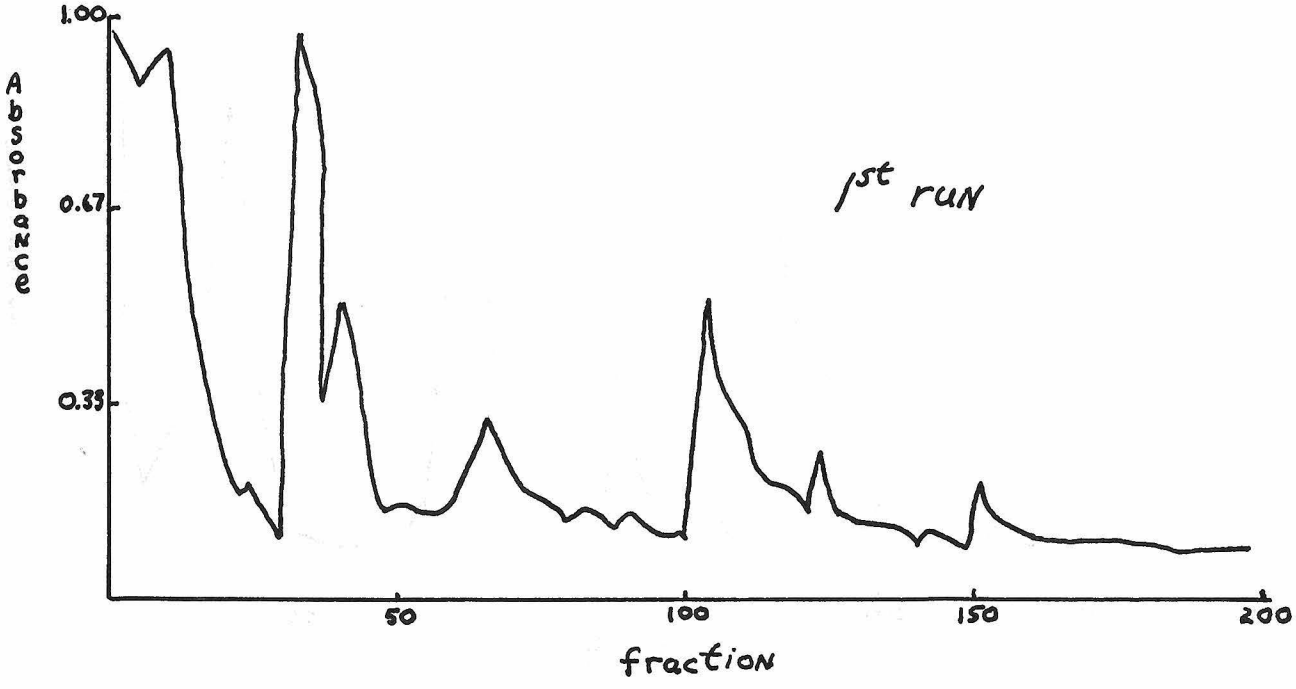
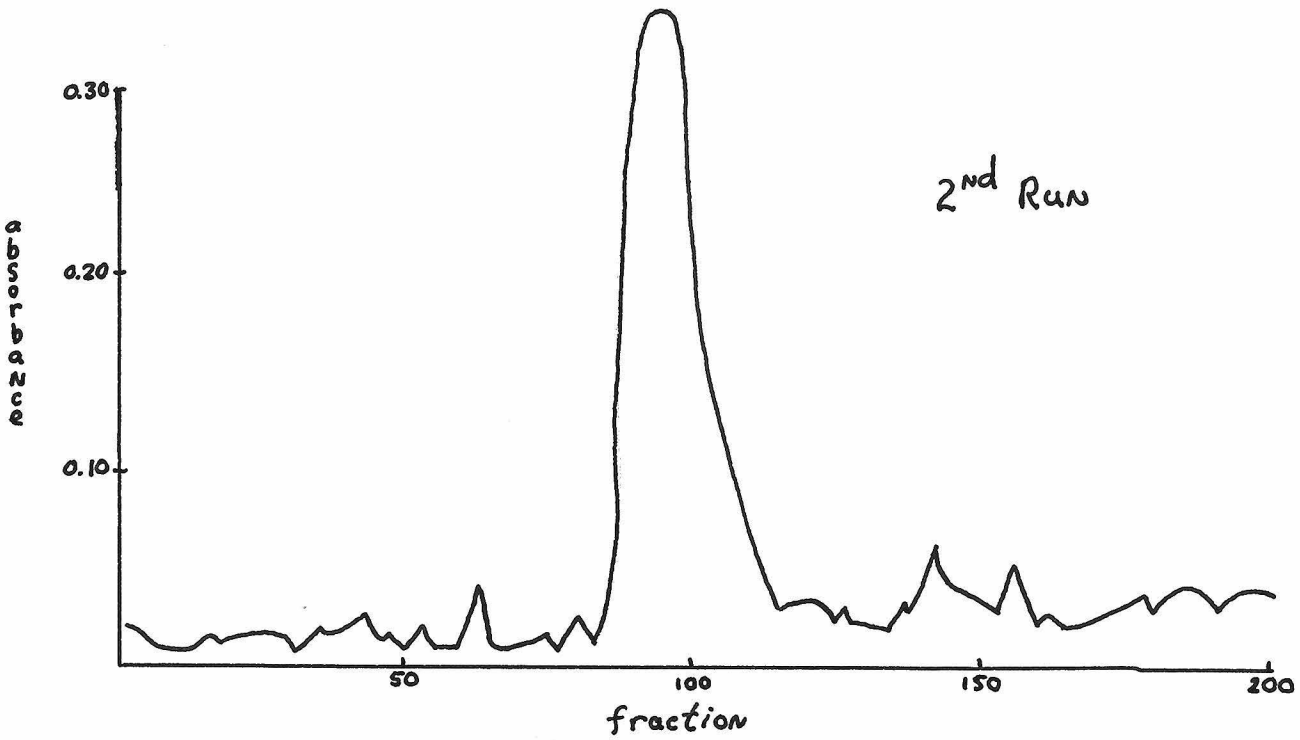
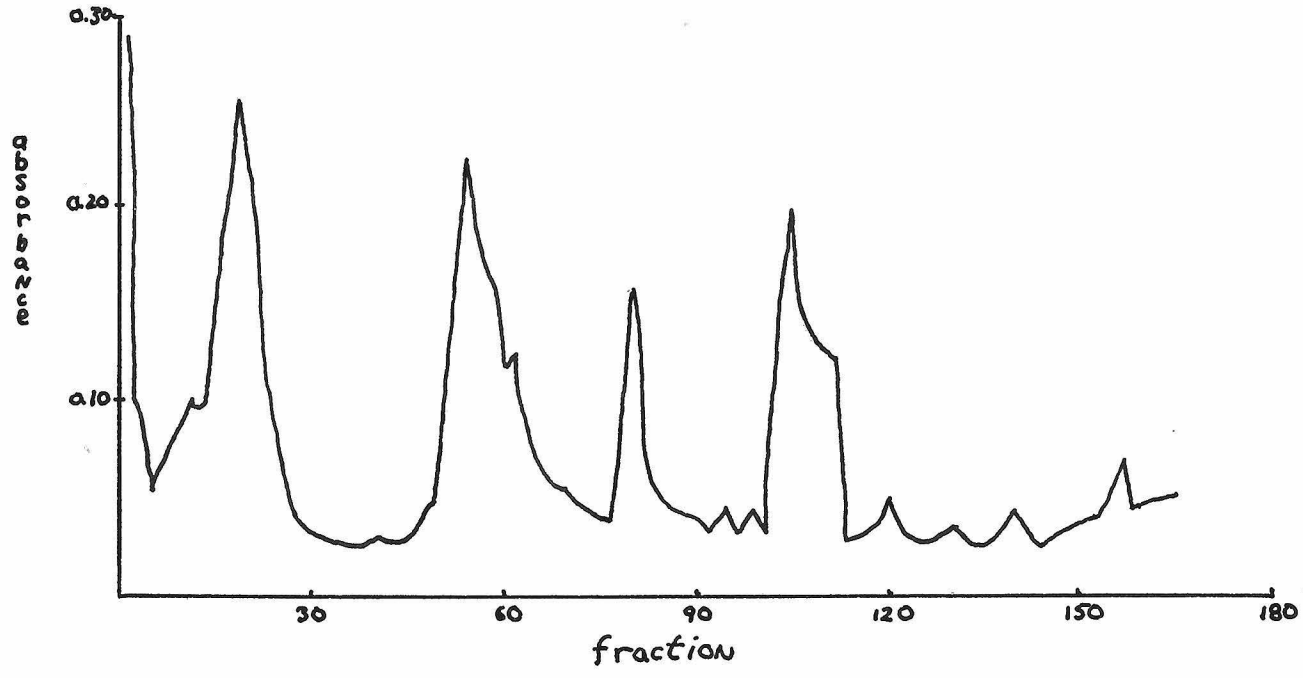
He was able to identify the peaks as the various polymers of N-acetylglucosamine by comparison of the optical rotations of the various fractions to values in the literature.

Unfortunately it proved difficult to reproduce the rather clear cut results obtained by Rupley. The analysis of the chromatographic fractions for reducing sugars gave the following results:





As can be seen the results were not only dissimilar to those of Rupley, but also inconsistent. The final fractions (134-170) from the first chitin hydrolysis were pooled, concentrated and rechromatographed on the same kind of column. The fractions were then retested for reducing sugars by the modified Somogyi method to give the following results:



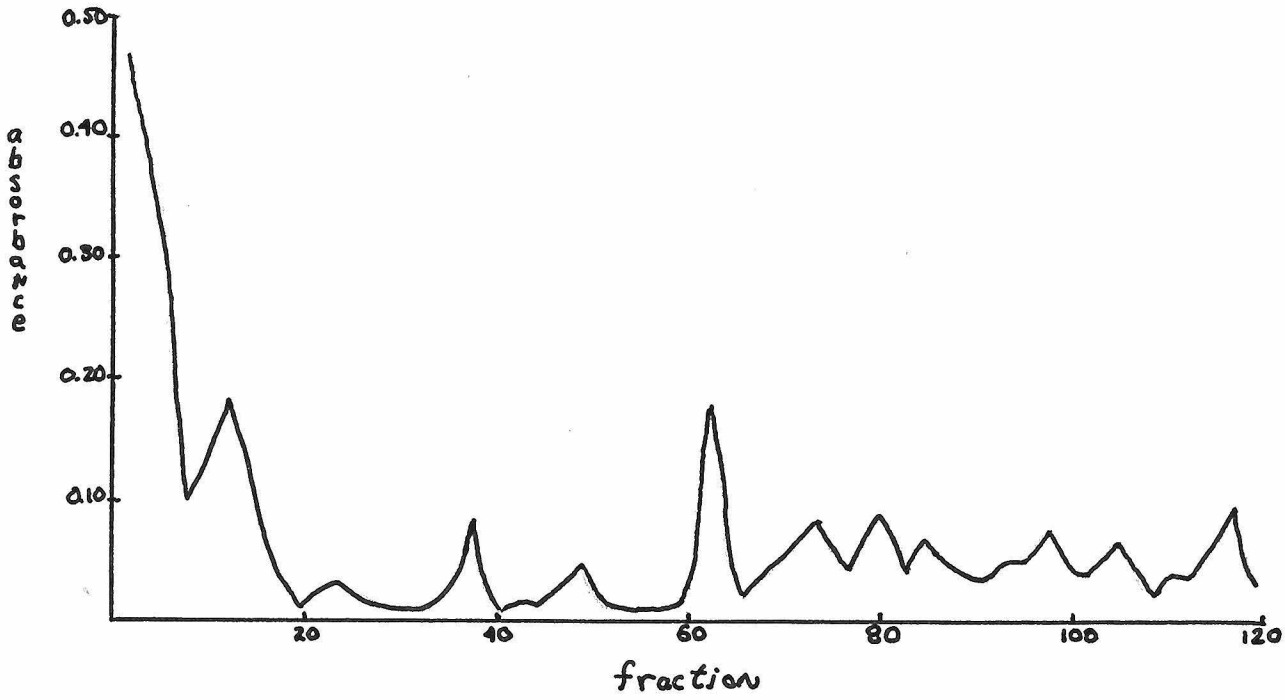
A further test for N-acetylamino sugars was performed using the spray reagents of Salton previously described. The appearance of purple spots corresponding to the peaks of the above chromatographic fractions also indicated the presence of N-acetylamino sugars.

The results of this work were certainly not so clear as had been hoped for. The results do seem to indicate, particularly for the first hydrolysis, that some sort of fractionation of the chitin polymers is taking place. With this in mind, this line of effort should be pursued, hopefully to attain better results. Perhaps some modification in the acid hydrolysis or in the chromatography may be necessary to obtaining better results.

Lysozyme Digestion of Micrococcus lysodeikticus Cell Walls

The other major portion of this work was involved with separating small saccharide components from Lysozyme digests of Micrococcus lysodeikticus cell walls. Some work was done with growing these cells, however purchased materials were eventually utilized. In a preliminary experiment the entire cells were incubated at 37° with lysozyme for several days. The digest was then placed in a dialysis tube and immersed in 300ml of cold water. The entire system was kept stirring in a cold room kept at 4°. After sixteen hours the dialysis was repeated with a fresh 300ml of water. The original 300ml

of dialysate were evaporated to a small volume and a thin layer chromatogram made using silica gell as the support medium and a 3:1:1 mixture of n-butanol, acetic acid and water as the solvent. An ammoniacal silver nitrate spray reagent was utilized for visualization. Several spots were obtained: a compact spot close to the solvent front and a long trailing spot about halfway to the solvent front and a long trailing spot from the origin. It was decided to do a charcoal-celite column chromatography of the dialysate. The column and solvent were prepared in the same manner as pre viously outlined for the chitin hydrolysis. The modified Somogyi method was again used for analysis of reducing sugars. The results of this turned out to be rather inconclusive as shown in the following graph:



Because of inability to identify the various fractions obtained it was decided that it was necessary to extract purified cell wall materials from the Micrococcus lysodeikticus prior to digestion with lysozyme.

This extraction was attempted by mechanical breakage of the cells. Portions of the cells were added to distilled water and number ten ballotini glass beads. The cells and beads were then ground with cooling for fifteen minutes in a Waring blender. The liquid was removed from the glass beads with a pipette and the beads washed several times with water, the washings being combined with the original liquid. This grindate was then centrifuged for 20 minutes at 3000 rpm to remove all intact cells. The supernatant from this was removed and centrifuged and 10,000rpm for 10 minutes to deposit the cell wall material. This material was washed with water and recentrifuged several times. The residue after the initial centrifugation at 3000rpm and the material obtained in the final centrifugation were examined under the electron microscope. Apparently a large number of cells remained unbroken. The original centrifugation yielded many intact cells plus some disintegration material. In the washed final preparation disintegration material with the appearance of cell surface was obtained. This material was then digested with trypsin and ribonuclease to remove any extraneous material. The remaining material was centrifuged out, washed twice with water and recentrifuged, then lyophilized.

The cell wall preparation was then digested for a period of 24 hours in an 0.05 molar ammonium acetate buffer at pH 6. A significant reduction in optical density indicated that digestion was occuring. The lysozyme digest was the dialyzed with 200ml of water for 24 hours followed by a further 150ml for 48 hours. The dialysate was then evaporated to dryness and dissolved in 2ml of water. A paper chromatogram of the material was performed with a descending solvent of n-butanol, acetic acid and water (3:1:1). The chromatogram was developed with the Salton spray reagent described in the first portion of this paper. The results obtained were in agreement with those of Salton.[6] Four distinct spots with R_f values of 0.0, 0.13, 0.28, 0.48 were obtained. A further chromatogram of the cell wall dialysate was also made using a solvent of pyridine-water (4:1). These results were also similar to those of Salton.[7] A single spot close to the solvent front plus trailing spots were obtained. Salton identified the fastest moving spot of the n-butanol,acetic acid, and water solvent chromatogram as a di-saccharide with a one to one proportion of N-acetylglucosamine - N-acetylmuramic acid. This fastest moving fraction was eluted, evaporated to dryness and redissolved in a small volume of water. A portion of this was added to a small volume of six normal hydrochloric acid and kept at 100° for 16 hours in a sealed tube to hydrolyze it. The hydrolysate was now chromatographed on paper with a pyridine-water solvent (4:1) and developed with

ninhydrin spray reagent. A comparison sample of glucosamine was also run on the chromatogram. A spot corresponding to the glucosamine was obtained, indicating that this fraction was probably the di-saccharide identified by Salton.

The results of this work make it appear that this is a workable method of preparing the di- and tetrasaccharides of n-acetylglucosamine - N-acetylmuramic acid. Work should now be done to see whether or not charcoal column chromatography can be utilized to separate the components of the cell wall lysate. This is necessary for larger scale preparations of these materials so that the proposed specificity and kinetic studies can be performed.

REFERENCES

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6. Salton, Biochim. and Biophys. Acta 40 (1960) 462

7. Salton, Ibid.