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Partial Characterization of LPSs from Rhizobium fredii USDA 205 Before and After NOD Gene Induction

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Partial Characterization of LPSs from *Rhizobium fredii* USDA 205 Before and After NOD Gene Induction

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BY

Bradley Lawrence Reuhs

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Abstract

The lipopolysaccharides (LPS) from Rhizobium fredii USDA 205 and its nod- mutant, HC 205, which has been cured of the 192-MDa symbiotic plasmid, were isolated by the phenol-water extraction method. The LPS from the wild-type strain which was grown in association with host-plant root extract or a flavone analog to a constituent of the root extract, which is required for the induction of at least a portion of the nodulation genes, was also isolated. The conditions required for the induction of the nod genes were determined by work with a nod- mutant of R. fredii USDA 201 which contains a Mu-lac insertion under the control of a nodulation gene promoter. The isolated LPSs were purified by column chromatography and partially analyzed by proton-NMR, gas chromatography (GC), colormetric assays, and polyacrylamide gel electrophoresis (PAGE) employing either sodium dodecyl sulfate (SDS) or 7-deoxycholic acid (DOC) as a detergent. Significant differences were seen in the DOC-PAGE banding patterns of the induced LPS samples when compared to the non-induced LPSs. The most important distinction being a relative reduction in overall LPS molecular weight by discreet increments for the induced LPS from USDA 205. This was accompanied by small molecular weight differences in the predominant bands of similar molecular weight. GC and colormetric analysis indicate that the polysaccharide

portion of the LPSs of all samples is composed predominantly of galactose and 2-keto-3-deoxyoctonic acid (KDO), a common constituent of gram-negative bacterial LPSs. There were some compositional differences between the induced and non-induced LPSs but the significance of these differences remains unclear at this time.

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INTRODUCTION

In the United States, the Soviet Union and other developed countries of the world the use of nitrogen fertilizer is both widespread and essential in nearly all aspects of agriculture. In order to meet the food demands of their growing populations these countries must continue to draw on both their financial and energy resources to manufacture and distribute these nitrogen fertilizers.

In much of the Third World, particularly Africa and South America, fertilizers may be prohibitively expensive or not available at all. Nitrogen is literally the limiting factor of agricultural production throughout the world at this time.

Amoung the essential food crops of the world are the ubiquitous members of the family Leguminoseae (beans, soybeans, clover, etc.). Members of this family are unique in their ability to form a symbiotic relationship with host specific members of the bacterial genus Rhizobium. Once the bacteria have established themselves in nodules on the roots of their respective hosts they exhibit the ability to convert (or fix) atmospheric dinitrogen into ammonia, a form of nitrogen which is easily metabolized by higher plants. The ability to fix nitrogen is found in other prokaryotes, such as Clostridium pasteurianum and Azotobacter sp., but only Rhizobium lives in this mutually beneficial circumstance, extracting photosynthate and providing metabolizable nitrogen, with higher plants. It is

precisely this relationship with such ethnobotanically important plants that has been the impetus for intensive study of Rhizobium and the process of nodulation (4).

Soybeans common to the american midwest (Glycine max) are nodulated by the bacterial species Rhizobium japonicum (now referred to as Bradyrhizobium japonicum). This bacterium has been the subject of much study. Recently a fast growing group of bacteria from the People's Republic of China which only nodulate the Glycine max cultivar Peking and Glycine soja have been characterized by Keyser et al (19). They have been designated Rhizobium fredii. In the bacterial strain Rhizobium fredii USDA 205 many of the genes necessary for the symbiotic process appear to be located on a 192-MDa plasmid (28). This has been demonstrated by the curing (i.e. removal) of the plasmid, which results in an organism that is unable to nodulate it's typical host. This Nod^- organism has been designated Rhizobium fredii HC 205 and has been found to be very useful, for comparison to the parent strain, in the analysis of symbiotic gene expression. The usefulness of this mutant strain for comparison and the fast growing nature of this species make this organism an ideal subject of study.

The process of nodulation is a multi-step process which involves the induction and repression of many genes in both the bacteria and the host plant. The initial event appears to be cellular recognition at the plant root. There is evidence that the host-symbiont recognition may be lectin

mediated. Lectins are proteins or glycoproteins which specifically bind to cell surfaces. It has been postulated that the lectins present at the plant root surface interact with polysaccharides at the surface of the Rhizobium cell to initiate nodulation (29). Various classes of Rhizobium cell surface polysaccharides have been described. These include exopolysaccharides (EPS), capsular polysaccharides (CPS), and lipopolysaccharides (LPS) as well as a variety of glucans (4,6).

Recognition is followed by attachment, possibly via the polysaccharide binding lectins, at an infectible root surface site. The first notable result of the plant-bacterial interaction is the curling of plant root hairs which leads to the enclosure of the attached rhizobia in a pocket of plant material. From within this pocket an infection thread (a tubular structure) develops and eventually enters the root cortical tissue carrying the infecting rhizobia within. From within the root cortex a nodule can then be formed (4).

One theory is that the bacteria are altered in some way, after contact with the host plant root or after association with a host plant root exudate. The alteration allows them to avoid detection and attack by the host plant defense system (14). The fact that the outermost polysaccharide of the cell wall is the molecular component of the bacterial cell most commonly recognised implies a change in the external components of the bacterial cell.

repeating oligosaccharide, known as the O-antigen, located at the external terminus (24). The O-antigen is that component of the LPS which is most often antigenic. The lipid portion is connected to the polysaccharide by a ketosidic bond involving 2-keto-3-deoxyoctonic acid (KDO). Previous studies of the Rhizobium leguminosarum cell wall have shown it to have a makeup similar to that of E. coli and Salmonella, in that there is a lipid-A, a polysaccharide portion and KDO (7).

One unique feature of the LPS of R. fredii that has been noted in previous studies is an unusually high KDO content when compared to the slower growing relative Bradyrhizobium japonicum, which also nodulates soy beans (9). The only other species of Rhizobium which has been reported to have large amounts of KDO is R. meliloti. Carlson and Yadav have found the LPS of R. fredii to contain about 8% KDO, which is similar to LPSs which lack the O-antigen portion of the LPS (9).

It is changes in the surface chemistry which have been postulated to be involved in determining the host specificity of Rhizobium (29). As stated before it is also possible that changes in surface chemistry are involved in the protection of the microorganism from the host plant defense responses and ultimately in the process of nodulation. In order to investigate the role of the LPS in the nodulation process it is important to examine the isolated LPS from the wild type parent strain as well it's

non-nodulating (nod$^-$) mutant, which is missing the plasmid known to contain many of the genes responsible for nodulation (25). This has already been done in part by Carlson and Yadav (9). In addition it is also important to examine both strains after association with the host plant root material which induces the nodulation genes.

Often in the study of genetics, particularly bacterial genetics, in order to understand the behavior of a gene, locate the gene, and isolate the gene product it is indispensable to have a complementary organism which is identical to the subject organism with the exception of a mutation in the gene of interest. One frequently used method of mutagenesis involves the insertion of a DNA element into a chromosomal or episomal gene, thereby disrupting the base-pair sequence and typically inactivating the gene product, either wholly or in part. This insertion may be the result of several mechanisms. For several years transposons have been used for mutagenesis. The transposon is similar to an insertion sequence (IS element) with the addition of a marker gene (such as the gene for kanamycin resistance found in the transposon Tn5) which allows for the selection of those organisms which have received the insertion (12).

For understanding the control of a particular gene there is no better mutagenic vector currently available than the mini-Mu- dI (Kan,*lac*) transposon (Mu-*lac*). In addition to the *Kan*^{R} gene for selection, the Mu-*lac*

also carries the operon (minus the promoter) for the production of B-galactosidase (B-gal), which can easily be quantified via a simple assay (11). The elimination of the operon promoter leaves the operon under the control of the host promoter for expression of the gene into which the Mu-lac has been inserted. Using this bio-technology Verma et al have demonstrated the inducibility of the nodulation genes in several species of Rhizobium (26). By the use of Mu-lac mutant organisms sent to us by Verma, we have been able to optimize the conditions for nod induction in our laboratory with the subsequent isolation of LPS, EPS and protein from induced cultures. These compounds have since been partially analyzed.

In addition to the use of conventional analytical techniques such as GC, NMR and colormetric tests, there have been a number of studies conducted using antibodies. Although these antibodies can be raised in a variety of small or large animals, the rabbit has proven itself to be one of the most effective media for this purpose. Rabbits are easy to maintain and produce a relatively large amount of blood sera compared to rats or mice; in addition they are hardy animals and can be injected with a wide variety of samples without suffering or damage. They may be injected with whole bacterial cells, which would result in the development of antibodies to many components of the cell, or islolated components such as polysaccharides or proteins may be used as antigens. The resultant antibodies can then be

used to test for subtle differences or similarities through the use of an enzyme-linked immunosorbent assay (ELISA) (2,13). In this assay the wells of a special polystyrene microplate are coated with a sample antigen, the antibodies are then applied, in the form of dilute antiserum, and they preferentially bind to the antigen. This is followed by the introduction of a goat anti-rabbit / enzyme complex which binds to all antigen bound rabbit generated antibodies. After the addition of the enzyme substrate a color will develop which can be used as a quantitative index of antibody preference for the sample antigen.

We have adapted the ELISA for use in our laboratory and we have begun the long and arduous process of generating, isolating, purifying, and testing antibodies for Rhizobium fredii USDA 205 and Rhizobium fredii HC 205 (both induced and non-induced) to aid in the study of these bacteria.

MATERIALS AND METHODS

ELISA

GENERAL PROCEDURE

The enzyme-linked immunosorbent assay (ELISA) was adapted for use in our laboratory from Fuhrmann and Wollum (13). The buffers and an outline of the procedure are described in Appendix 11. Whole cells used as antigens were first removed from working slants with ca. 3mL of 0.85% NaCl. They were then heat treated for 15 minutes in a 100°C water bath. After cooling to room temperature the antigen suspensions were diluted to an optical density of ca. 0.5 absorbance units at 600nm with coating buffer. 0.1mL of this suspension was then delivered to the appropriate wells of a polystyrene microplate (Cooke microtiter; Dynatech Laboratories). The plates were then incubated overnight at 4°C or for one hour at 37°C. Excess antigen was shaken out and the plates were washed three times with PBS-Tween by filling each well individually with a pipette. The first wash was emptied immediately while the last two were left for 5 minutes. Rabbit anti-Rhizobium antisera was then added to each well (0.1mL) after serial dilution in PBS-Tween. Dilution range varied with each experiment. The plates were then incubated for 1 h at room temperature. The washing procedure was then repeated and 0.1mL of goat anti-rabbit globulin / alkaline phosphatase conjugate (Sigma Chemical Company) diluted in

PBS-Tween was added to each well. The plates were covered and incubated for 3 h at 37°C or overnight at 4°C. The washing procedure was then repeated. A substrate solution consisting of p-nitrophenyl phosphate diluted in substrate buffer was added to each well (0.1mL). The plates were left at room temperature to develop. After sufficient yellow color had developed the reaction was stopped with addition of 50uL of 3M NaOH solution. The contents of each well were removed to test tubes containing 2.0mL H_2O. The absorbance at 405nm was then read on an LKB Ultraspec 4050 and the results were plotted on a graph. Although the incubation periods may vary, the times given in the Appendix were found to be the most effective and practical.

RABBIT ANTI-RHIZOBIUM ANTIBODY DEVELOPMENT

The cells were grown at room temperature, on a shaker, in 40 mL of a typical yeast extract medium (see Appendix 14). When they reached an O.D. of ca. 1.85 at 620nm (R_f USDA 205, 1.75 at 620nm and R_f HC 205, 1.98 at 620nm) they were harvested by centrifugation at 9150xg in sterile tubes and washed in sterile 0.85% NaCL. After repelleting the cells were resuspended in 10mL of PBS (10mM $NaPO_4*7H_2O$ / 150mM NaCl). This was used as the stock for the inoculation of the rabbits. All bacterial cultures were tested for purity by streaking on plates of Difco Bacto Nutrient Agar and Gram stain analysis.

The rabbits received injections in the marginal ear

vein on a regular schedule as indicated in the appendix. Seventeen days after the initial injection the rabbits were killed and the blood removed directly from the heart and placed in test tubes. The blood was allowed to clot at room temperature for two hours before being stored overnight at 4°C. The sera portion of the blood was carefully removed with a Pasteur pipette and the clots were centrifuged at 2000rpm to isolate the remaining sera. The supernatent was added to the other sample and stored in 1mL aloquots at -20°C.

PURIFICATION OF THE RABBIT ANTI-*RHIZOBIUM* VIA ADSORPTION

In order to increase the specificity of a particular antiserum it was often necessary to remove common antibodies through a process of adsorption. To accomplish this a volume of antisera was combined with the cells from a cross reacting strain. The cells were removed from the working slant with 2-3mL of 1M NaCl, they were vortexed to seperate clumps and remove capsular material. The suspension was then centrifuged at 13,000rpm for 30 minutes. The supernatent was discarded and the pellet was combined with the antisera to be purified. The mixture was incubated at 37°C for three hours. The cross reacting bacteria agglutinated and settled out and the supernatent was removed. The process was repeated on the supernatant until there were no more crossreacting antibodies as indicated by

a lack of agglutination.

Although the procedure described above is adequate for small quantities (1-2mL) of antisera or for bacterial strains whose cross reaction is limited, it proved to be too slow and much too cumbersome for large quantities of heavily crossreacting antisera such as those developed against R. fredii HC 205 and USDA 205. For these it was necessary to purify 10mL volumes of antisera by growing 40mL cultures of the crossreacting strain to an O.D. of ca. 1.5 at 620nm. The cells were then pelleted and washed 3 times with saline. The final pellet was then mixed with the antisera and the whole volume of mixture was incubated as described. After the adsorption process was completed the purity of the antisera was tested by performing an ELISA. All cultures were tested for purity as described above.

ISOLATION OF LIPOPOLYSACCHARIDES

Bacterial lipolysaccharide (LPS) was isolated by the modified hot phenol/water method (8). A large batch of cells was grown in either an 8.0L bottle with sterile air bubbled through or in sixteen 500mL cultures on a shaker. The cells were harvested by centrifugation (20 minutes at 8000 rpm) and checked for purity on Nutrient Agar and by Gram stain. A suspension of bacteria was subjected to disruption by blending in a Waring blender followed by sonication. The suspension was treated with lysozyme, RNase

and DNase after which it was heated and combined with hot phenol. Subsequent centrifugation resulted in the division of the mixture into two layers, the phenol layer which contained bacterial discard and the water layer which contained the LPS. After removal of the water layer the phenol layer was re-extracted using fresh hot water. The combined water layers were dialyzed against water and finally lyophilized (see Appendix 13 for details). The LPS was purified from the water layer by column chromatography, first on a Sepharose 4B column of 40cm in length by 6cm in diameter and later on another 4B column of 110cm in length by 1.8cm in diameter. The length of this second column provides for better resolution. The desired peaks, as detected by qualitative colormetric assays for KDO (31) and hexose, were then collected, dialyzed against water and lyophilized. Details of these colormetric assays may be found in Appendices 5 and 6.

QUANTITATIVE COLORMETRIC ASSAYS OF LIPOPOLYSACCHARIDE SAMPLES

A 1.0mg/mL solution of freeze-dried LPS in water was made for all samples. Each LPS sample was then analyzed by quantitative colormetric assays for KDO (31), uronic acid (3), pyruvyl groups (18), acetyl groups (15), and hexose (by the anthrone method). Each assay included a set of standards for comparison. The results of the assays were

then subjected to linear regression analysis and a percent of total mass was calculated for each. Details of the assays may be found in Appendices 5-9.

POLYACRYLAMIDE GEL ELECTROPHORESIS OF LPS SAMPLES

The LPS samples were analyzed by polyacrylamide gel electrophoresis (PAGE) as described in appendix 1. A 1.0mg/mL solution of each sample was prepared; 30uL of this solution was lyophylized then redissolved in 25uL of sample buffer (see appendix 1) and heat treated in sealed tubes at 100^{o}C. The samples were allowed to cool, then run on a 12% to 15% polyacrylamide gel containing either sodium dodecylsulfate (SDS) (21), Triton X100, or deoxycholic acid (DOC) (20) as a detergent. In some cases a gradient gel from 10% to 20% acrylamide was used. Usual running time was about 3 hours at a constant current of about 20ma on 12cm Hoefer Scientific Instruments SE 500 Slab Unit for SDS and Triton gels. For DOC gels the current was kept constant at 18ma through the stacking gel and 25ma through the running gel. The DOC gels were run on either a 16cm Hoefer Scientific Instruments Sturdier SE 400 Slab Unit (about 3.5 hours running time) or a Sturdier SE 420 32cm Slab Unit (4.5-5.5 hours running time). When the bromphenol blue dye reached the bottom of the gel the gel was removed from the apparatus and stained for LPS by the silver staining method of Tsai and Frisch (30). See appendix 2. Stained gels were

stored in Saran wrap at 4°C. All gels were calibrated by the inclusion of a well containing LPS from wild type Salmonella and LPS from a rough mutant of Salmonella.

PROTON NUCLEAR MAGNETIC RESONANCE (NMR) ANALYSIS OF THE LIPOLYSACCHARIDES

Proton NMR studies were performed on a General Electric QE-300 NMR at the University of Illinois (Champaign-Urbana). The preparation of the samples consisted of dissolving 10-20mg of freeze-dried sample in 2.0mL deuterium oxide, lyophylization, and redissolving of the sample in another 2.0mL of D_2O. The more concentrated the sample the better the resolution. For those samples which produced a relatively clear spectra, 300 aquisitions proved adequate; however some samples required 600 or 900 aquisitions. The sample spin rate was maintained between 20 and 30 RPS.

ANALYSIS OF LPS BY GAS CHROMATOGRAPHY

Gas chromatography studies were performed on acetylated samples. Samples were subjected to hot acid hydrolysis followed by reduction with sodium borohydride. The sample was then treated with methanol and acetic acid to remove the $NaBH_4$. The reduced sample was transformed into alditol acetates by treatment with acetic anhydride and pyridine.

The alditol acetates were then extracted (separated from salts formed during the treatment) with chloroform. After evaporating the $CHCl_3$ by blow drying with filtered air, the sample was dissolved in dichloromethane and analyzed on a Hewlett-Packard 5890A Gas Chromatograph interfaced with an Apple IIe computer. The column used was a 15 meter SP2330 fused silica capillary column. The run was from 190°C to 240°C at a rate of 10° per minute, followed by a 15 minute hold at 240°C (see Appendix 10 for details) (1).

For samples with a high KDO content a special procedure was used in the acetylation process (appendix 10) (32).

INDUCTION ANALYSIS OF MU-*LAC* INSERTION MUTANTS

In order to optimize the induction conditions to be used in the analysis of *Rhizobium fredii* USDA 205 and it's nod^- mutant *Rhizobium fredii* HC 205, it was first necessary to examine the induction phenomenon quantitatively. This was accomplished by the use of a closely related bacterial strain which contains a Mu-*lac* gene insertion that resulted in the elimination of the ability of this strain to nodulate its natural host.

Three mutant (nod^-) strains of *Rhizobium fredii* USDA 201 were sent to us from the laboratory of Desh Pal S. Verma (McGill University, Montreal, Quebec, Canada). The strain *Rhizobium fredii* USDA 201::Mu-d (KAN,*lac*) was innoculated into 40mL of yeast extract media (YEM) which

contained 50ug/mL of kanamycin. This culture was grown at 26°C on a shaker to an O.D. of 0.57 at 600nm. From this flask 10mL of culture was used to inoculate two duplicate sets of two flasks. Each sterile 50mL flask contained 10mL of YEM with 50ug/mL of Kanamycin. The experimental flasks also contained 1mg/mL of root extract, for a final concentration of 0.5mg/mL after addition of the starter culture. The time of inoculation of the experimental and control flasks was taken to be time zero (T=0). At this time the first sample (1.5mL) was removed from each flask and subjected to the B-galactosidase assay described in Appendix 15. Samples were then taken after three hours (T=3), six hours (T=6), eight hours (T=8), ten hours (T=10), twelve hours (T=12), and twenty-four hours (T=24). The results were plotted as the activity units of B-galactosidase versus time. The growth curve was also followed (O.D. at 600nm). A subsequent experiment was performed in the same manner with a five fold increase and a five and twenty-five fold decrease in the root extract concentration (26).

The induction of a large culture (4L) was tested using large bottles of YEM broth with sterile air forced through an air stone to provide oxygen. The root extract was provided to the experimental bottle at 0.5mg/mL and kanamycin was added to both experimental and control bottles at a final concentration of 50ug/mL.

The effect of apigenin on induction of the nodulation

genes was also tested using a similar experiment. Four flasks containing 40mL YEM were innoculated from a starter flask in stationary phase. The negative control flask contained the medium and 50ug/mL of kanamycin, while the positve control contained the kanamycin as well as 0.5mg/mL of root extract. The experimental flask contained the kanamycin plus 3ug/mL of sterile apigenin. In addition there was an inhibition test performed with kanamycin and 100ug/mL of apigenin. Samples were assayed as before for B-galactosidase activity. This last experiment was repeated using all three strains of Rhizobium fredii USDA 201::Mu-lac.

For a summary of all bacterial strains used in the writing of this paper see table 1.

STRAIN	DESCRIPTION
Rhizobium fredii USDA 205	Wild-Type (nod+)
R. fredii USDA 205 (IND)	W-T after nod induction
R. fredii HC 205	Lacks sym plasmid (nod-)
R. fredii USDA 201 11G2	Mu-lac mutant (nod-)
R. fredii USDA 201 21D5	Mu-lac mutant (nod-)
R. fredii USDA 201 22G1	Mu-lac mutant (nod-)
Salmonella minnesota	Wild-Type
S. minnesota R60	Ra mutant, lacks O-antigen

TABLE 1. Bacterial strains used in the course of this study. Only the LPS from Salmonella minnesota was used.

RESULTS

ADAPTATION OF ELISA AND DEVELOPMENT, ISOLATION, PURIFICATION AND TESTING OF ANTIBODIES

ELISA

After performing the ELISA several times the procedure outlined in methods was found to be the most reliable. As can be seen in figure 1 the ELISA demonstrates a very definite specificity when using two different species of rhizobia (R. japonicum vs. R. trifolii). However when using two different strains of the same species (R. japonicum 123 vs. R. japonicum 110) the differences in specificity are not always so dramatic (figure 2). In this case it is necessary to purify the antiserum by adsorption in order to obtain strain specific antiserum. The result (figure 3) is a very definite increase in specificity although it is accompanied by approximately a three-fold drop in activity. The specificity however is the important aspect. Note that the optimum dilution for specificity is about a 1/800 (log at 2.9) dilution of the antiserum, this proved to be the optimum dilution in many of the ELISA tests performed, particularly those that required little or no adsorption purification. After lengthy adsorption purification the optimum dilution drops to a range of 1/25 to 1/100 (log at 1.4 to 2.0) due to the agglutination and removal of many of the antibodies from the antisera.

ANTISERA

The development of the antibodies against Rhizobium fredii USDA 205 and Rhizobium fredii HC 205 yielded ca. 44mL of sera from each rabbit. A portion of each antiserum was purified by adsorption and an ELISA was performed using both adsorbed and unadsorbed antisera. The unadsorbed antiserum is that which is used as extracted from the rabbit and the adsorbed antiserum is that which has been incubated in the presence of the crossreacting strain of bacteria until common antibodies have agglutinated and settled out completely. An example of this purification process is the incubation of the antiserum produced by the rabbit which was injected with USDA 205 in the presence of HC 205 bacteria. This process may be repeated until there is no further agglutination. The results of an ELISA performed with antisera to HC 205 and USDA 205 which has been purified in this fashion (shown in figure 4) show some specificity by the purified antisera, though very little (only about a three-fold difference in specificity for USDA vs. HC as compared to a forty-fold difference in figure 1 and a ten-fold difference in figure 3). This is to say that there is minor preferential color development in the microtiter wells which have been coated with the USDA 205 bacteria and incubated with USDA 205 antisera that had been treated with HC 205 bacteria.

At the time of this work we were not yet aware of the necessity for induction of the nodulation genes. Since the

difference between the two strains (HC 205 and USDA 205) is the lack of the symbiotic plasmid in HC 205 it is not at all surprising that they do not demonstrate a significant difference in antigenicity without prior induction of the culture. It is known that at least some of the genes on the plasmid are not active without induction (27) and therefore any gene products from these genes that might be antigenic are not accounted for in the antiserum to USDA 205. Generation of antibodies to induced cells was performed in the same fashion as other antibody development. The rabbits were innoculated with cells that were grown in the presence of apigenin as per induction analysis (below). The injection schedule was the same as for the other rabbits. When an ELISA was performed on the induced antibodies as well as the USDA non-induced antibodies there was no color development in either the experimental wells or the control wells. It is not clear if the problem was with the ELISA buffers in stock or the antisera obtained from these rabbits. Due to time constraints the test could not be repeated. However, the results already obtained will be important in the future analysis of the data from the induced antigens.

INDUCTION ANALYSIS

The results of the initial induction experiments with the *Rhizobium fredii* USDA 201::Mu-*lac* 11G2 Nod^- mutant

strain show B-galactosidase activity (indicating B-gal production) in the flask containing root extract that is nearly ten-fold higher than the control (figure 5). The activity is calculated in activity units according to the following equation;

$$\text{ACTIVITY UNITS} = \frac{\text{O.D. @ 420nm}}{\text{T x V x O.D. @ 600nm}}$$

where T equals the time of incubation of the sample taken (see appendix 15), and V equals the volume of sample taken.

There is a lag time of approximately three hours as shown in figure 5 after which the activity in the induced culture rises sharply. These data are very similar to those in the Verma paper (26), although more data points after the 12 hour time period were shown in that work which indicate a precipitous drop in activity for a short time followed by a return to the highly induced levels (26). For our purposes our data are sufficient to dictate protocol for the induction of a larger batch with the aim of isolating a workable quantity of LPS from Rhizobium fredii USDA 205 and R. fredii HC 205. In order to verify that the concentration of root extract used is optimal an experiment of varied concentration was designed. The 0.5mg/mL concentration of root extract used in the previous experiment is considered the positive control and again there is a flask without root extract as the negative control. A five fold increase in the extract concentration is introduced into one flask as well as a five and a twenty-five fold decrease in two other flasks. The results

(data not shown) indicate that the optimal concentration lies between 0.5mg/mL and 0.1mg/mL. There is some inhibition at the higher (2.5mg/mL) concentration in the first few hours of sampling. The twenty-five fold decrease (0.02mg/mL) in concentration does not show an activity of more than twice that of the negative control.

Following this experiment, which uses cultures of only 20mL, an experiment was set up with 4L cultures in large (6L) bottles. In addition to the larger volume of medium in which the cells are grown, these bottles are aerated by forced filtered air and not by shaking as are the smaller flasks. The induced culture does demonstrate B-gal production but not at the levels desired (data not shown). It is possible that there is not as much bacterial interaction with the inducing agent in the absence of shaking. Another possibility is that the forced filtered aeration system employed with the large bottles maintains an O_2 level which is inhibitory towards induction. It appears that the best results can be obtained from 500mL cultures that are grown in 1000mL culture flasks on a shaker.

Information from other sources (27) indicate at this time that the inducing agent in clover root extract might be a flavone, apigenin. To test this in soybeans an experiment was conducted using the 0.5mg/mL concentration of root extract as the positive control and a flask with no inducing agent as the negative control. The experimental flask contained filter sterilized apigenin at a concentration of

3ug/mL. An inhibition test was also run using a 100ug/mL concentration of apigenin. The results show a B-gal activity in the 3ug/mL apigenin culture that is nearly identical to that in the root extract control (140 activity units for 3ug/mL apigenin at highest activity level as compared to 127 activity units for 100ug/mL at highest activity level). Growth inhibition is dramatically demonstrated in the 100ug/mL apigenin test flask which shows no real growth throughout the experiment. Upon the completion of the experiment cells, from this inhibited culture were streaked out on YEM plates with no apigenin to find out if the apigenin at such high concentration is fatal or merely inhibitory. The transfered cells grow on the plates without apigenin, indicating that the high concentration of apigenin is not fatal.

The similarity in B-gal production by this Mu-lac mutant in the presence of either root extract or apigenin demonstrates that either one may be used for the induction of large batches for the isolation of symbiotic gene products. To further simplify the procedure for large scale induction of R. fredii 205 or 201, an experiment was performed that compared the level of induction in a culture containing 3ug/mL apigenin which had been filtered sterilized with a culture containing an identical concentration of apigenin which had been autoclaved along with the medium. The results indicate that the sterilization process has no significant effect on the ability of the

compound to induce the lac gene.

In this experiment two other Mu-lac mutants that were sent to us by Dr. Verma were also subjected to induction analysis via both root extract and apigenin. Surprisingly neither shows induction of any significance. Since none of the three strains had been tested by Verma's lab it seems a fortuitous decision to use the 11G2 strain from the outset. The object of the experiment is not to analyze the behavior of the 201 mutants, but rather to confirm or support interchangability of apigenin and root extract. To this end the lack of induction of either of the other strains does not refute the conclusion that the two inducing agents may be used interchangably.

LIPOPOLYSACCHARIDE ISOLATION AND PURIFICATION

The LPS from Rhizobium fredii USDA 205, induced and non-induced, and Rhizobium fredii HC 205 were isolated as described in the methods section. The final lyophilized weights of the isolated LPSs (non-induced) were 176.0mg for Rf USDA 205 and 323.8mg for HC 205. They were subsequently purified by column chromotography, first on a large volume Sepharose 4B column and then on a second, higher resolution, Sepharose 4B column. The first purification step was followed by a hexose assay and a KDO assay. The results are shown in figures 6-8. The second peak in each case was combined, reduced in volume and freeze-dried. It was this

peak that was then run through the high resolution 4B column. A KDO assay was then performed on the fractions collected. These assays resulted in large peaks (shown in figures 9 through 11) which exhibited a natural separation point at a shoulder in the profiles of USDA 205 and HC 205 and a rather symetrical peak in the profile of the USDA 205 (IND). The fractions from USDA 205 and HC 205 were separated into two samples at the respective shoulders. Although the profile for USDA 205 (IND) did not exhibit a point of delineation, for comparative purposes it also was divided into two separate samples at the same place as the non-induced USDA 205.

A method for further separation was tested using a formate column buffer at pH 3.3, but it proved unsuccessful.

Due to the high relative quantity of KDO in the samples there is the danger of acid hydrolysis of the polysaccharide with this slightly acidic buffer. The lability of the LPS hinders attempts to separate the fractions further. However the separation achieved was more than adequate for initial GC and NMR analysis as well as for the various quantitative colormetric tests performed. Several alternative methods of improving separation have been considered and will be tested in the future. They will be discussed shortly.

The LPS I and LPS II fractions, so designated by positions in the 4B column profile (see figures 9 and 10), from each organism were reduced in volume and freeze-dried. lmg/mL solutions were then used as the stock solutions for

further analysis; the remainder was stored at -21°C.

COLORMETRIC ANALYSIS OF PURIFIED LPS I AND LPS II ISOLATED FROM RHIZOBIUM FREDII USDA 205 (IND AND NON-IND) AND RHIZOBIUM FREDII HC 205

Quantitative colormetric tests were performed on all LPS samples isolated. The tests included assays for acetyl groups (ACE), pyruval groups (PYR), KDO, uronic acid (URO), and hexose (HEX). The results are expressed in percent of total weight as calculated from a standard curve generated from the assays of standard sugars followed by linear regression analysis. Details of each assay procedure are found in appendices 5-9. The results of the tests are contained in the following table;

LPS SAMPLE	% TOTAL WT.				
	P Y R	A C E	U R O	K D O	H E X
HC 205 LPS I----------	2.2	0.8	4.4	53.8	29.2
HC 205 LPS II---------	1.8	0.7	2.0	41.2	38.0
USDA 205 LPS I--------	1.7	1.0	3.5	52.4	16.0
USDA 205 LPS II-------	2.1	0.9	2.2	52.2	25.7
USDA 205 (IND) LPS I--	0.4	0.8	1.2	43.3	27.9
USDA 205 (IND) LPS II-	0.9	0.8	5.1	51.7	29.2

TABLE 2. Relative percent composition of LPS samples as shown by colormetric analysis (for details see text).

NMR ANALYSIS OF LPS

Strict interpretation of the results from NMR analysis of the LPS samples is very difficult due to the complexity of the samples and the lack of documented data in this area. However the spectra do give some useful information (the spectra are located in figures 12 through 17).

The proton-NMR spectra for HC 205 LPS I and LPS II show only one difference when compared to one another. The peak at ca. 1.4 PPM is somewhat reduced in height and area in the LPS II spectrum. This peak is often associated with the presence of a pyruval group. This difference may not be intrinsically significant because results of other experimentation (see PAGE results and discussion) have implied that the LPS II may simply be LPS which was fragmented during the isolation or purification processes. The peaks at ca. 2.0 PPM and ca. 2.5 PPM are indicative of the methylene protons of KDO (see figure 12). These peaks appear as doublets or triplets in the spectra from HC 205 LPS I and LPS II. The KDO methylene carbon (C-3) peak at ca. 2.0 (from the axial proton $<C\text{-}3/H_a>$) which appears as a triplet is actually a doublet of doublets which is due to the nearly identical coupling constants of the equatorial proton on the same carbon ($C\text{-}3/H_e$) and the axial proton on the adjacent carbon ($C\text{-}4/H_a$). The peak at ca. 2.5 is from the axial proton on the methylene carbon and appears as a doublet because the coupling constant between this proton

and the C-4/H_a proton is much smaller and consequently the shift is much smaller.

The same pattern exists for the spectra from USDA 205 LPS I and LPS II, although the peak at 1.4 PPM is nearly the same size in both of these spectra. The series of peaks between 3.4 PPM and 4.4 PPM are due to the various protons attached to the ring structures of the various sugars in the sample. The polymeric nature of these samples is responsible for the complexity of these spectra. Downfield from the large group of peaks, at about 4.8 PPM, is a peak due to the H_2O in the solvent. At ca. 5.15 PPM is a peak which appears to be the result of the anomeric protons of galactose.

The spectra for the LPS from induced cultures of USDA 205 show the region betwwen 1.0 PPM and 3.3 PPM and do not appear to differ significantly from the spectra of the four non-induced samples. The small peaks at ca. 1.2 are due to contamination by TEA. The peak at 2.0 PPM appears somewhat more complex than in the non-induced spectra. The reason for this is not clear, however it may be that there is some modification of the KDO or there might be a difference in the arrangement of the polymer.

LIPOPOLYSACCHARIDE SAMPLE ANALYSIS BY GAS CHROMATOGRAPHY

The samples were prepared as described in the methods and appendix 10. The standard run failed to give a KDO peak for quantification of KDO in the samples, however a separate run of standard did give the retention time for KDO. This provides some very important data.

The GC profiles are shown in figures 18 through 23. Inositol was added to each sample as an internal standard.

The xylose (XYL) and rhamnose (RHA) peaks do not appear to be significant and remain minor throughout the profiles. The peak between 790 and 800 seconds is the peak for monomeric KDO as determined by separate analysis. The peak at 267 has been shown by GC-Mass Spec analysis to be a methyl-hexose, although the exact type is not known (5). Table 3 contains the quantitative data obtained from the GC analysis.

Close inspection of the profiles from 750 seconds to 875 seconds in retention time reveals the most interesting feature of these data, a sharp reduction in the relative peak size for KDO in the profiles from the induced samples when compared to the profiles from USDA 205. This reduction in the KDO peak is in concert with the appearance of a new and very significant peak at 860-865 seconds in the USDA 205 (IND); it is most pronounced in the LPS II sample. Although this peak is present in the profiles from USDA 205 and HC 205, it is at levels which are much reduced and it

LPS SAMPLE	% TOTAL WT.					
	RHA	XYL	MAN	GLC	MHX	GAL
HC205LPSI----------	0.1	---	1.1	2.9	2.4	17.6
HC205LPSII----------	0.2	0.1	1.7	2.6	3.3	25.6
USDA205LPSI---------	0.1	0.0	0.7	1.9	2.4	20.1
USDA205LPSII--------	0.2	---	0.8	0.6	2.8	21.7
USDA205(IND)LPSI----	0.2	0.2	1.0	4.5	3.1	15.2
USDA205(IND)LPSII---	0.1	0.1	1.0	4.5	2.3	18.9

TABLE 3. Reative percent composition of LPS samples as shown by gas chromatographic analysis (GC). The abreviations used are: RHA, rhamnose; XYL, xylose; MAN, manose; GLC, glucose; MHX, methylhexose; and GAL, galactose. All percent compositions were generated from standard sugars exept the methylhexose percent which was calculated by comparison to glucose.

appears to be associated with another peak of nearly the same retention time. In the profiles from all six samples there is a peak at about 760 seconds which is consistently similar in height and area to the KDO peak. In the induced samples this peak is reduced by the same magnitude as is KDO. Although these peaks have not been identified at this time, there are three lines of evidence that suggest that they are closely related to KDO: 1) The retention time for these peaks are relatively close to that of KDO. The peaks for the various hexoses all appear before 475 seconds in these profiles, and as determined by separate analysis (data

not shown), a common heptose would show a peak in the area of 600 seconds. Therefore the fact that these peaks are separated by about 50 seconds in retention time from KDO indicates that they are probably eight carbon sugars. 2) The colormetric results indicate that the percent composition of KDO (or a related compound that would test positive in the procedure used) is approximately the same in both the induced and non-induced samples. 3) The total area of the four peaks from 760-865 seconds remains realtively constant in all six profiles; there is simply a shift in proportion between the induced and non-induced samples.

POLYACRYLAMIDE GEL ELECTROPHORESIS OF LIPOPOLYSACCHARIDES FROM USDA 205 (INDUCED AND NON-INDUCED) AND HC 205

The LPSs from Rhizobium fredii USDA 205 (induced and non-induced) and Rhizobium fredii HC 205 were analyzed by polyacrylamide gel electrophoresis (PAGE) as described in the materials and methods section of this paper. The specific procedures may be found in appendices 1 and 2. A 13-15% acrylamide gel was used for LPSs and the finished gel was silver stained by the method of Tsai and Frasch (30). A reproduction of one gel may be found in figure 24. As with the NMR and GC results there is a notable qualitative difference between the resultant pattern of the USDA 205 sample and the induced USDA 205 sample.

In figure 24 lanes 1 and 2 contain USDA 205 LPS I and

LPS II, respectively. As in previous work with these samples the LPS II shows little or no staining (9). This is also the case for lanes 5 and 6 which contain HC 205 LPS I and LPS II, respectively. However the lanes containing induced USDA 205 LPS I (lane 3) and LPS II (lane 4) both show staining, with lane 4 staining slightly darker. This is not an anomolous finding when it is taken into consideration that the column chromatography profiles for these samples (figures 9 & 10) show a natural shoulder for USDA 205 and HC 205 but not for the LPS from the induced culture. These chromatography profiles are similar to those of previous work (9) in the case of the non-induced samples and of work performed more recently in the case of the induced sample (10).

Lane 7 contains the LPS from Salmonella which is used as a known control. A mixture of LPS from wild type Salmonella and a mutant are used. The wild type Salmonella produces an LPS that contains an O-antigen made up of multiples of identical units of polysaccharide. Any given cell may produce O-antigen containing various numbers of these repeating units, which upon isolation, purification and analysis by PAGE give a characteristic ladder-like banding pattern. The mutant Salmonella strain is lacking these repeating units and shows one large band of relatively low molecular weight.

Lane 8 is the same as lane 4, except that it contains double the amount of sample material.

As seen in figure 24 USDA 205 LPS I (lane 1) and HC 205 LPS I (lane 5) show a similar banding pattern, though HC 205 appears to contain more sample material. Both USDA 205 and HC 205 are notably different from the lanes which contain the induced samples, lane 3 (USDA 205 LPS I <IND>) and lane 4 (USDA 205 LPS II <IND>).

As previously noted the original LPS sample from the induced USDA 205 was separated into two fractions for comparative purposes only. From figure 24 it appears that this separation was unnecessary. The only visible difference between the USDA 205 LPS I (IND) and the USDA 205 LPS II (IND) is the intensity of the bands. The separation of the non-induced samples was based on a shoulder which appeared in the profile generated after purification by column chromatography. From the evidence of PAGE, in which the LPS IIs of the non-induced samples run to the bottom of the gel, and other data, which show either no difference between the LPS I and LPS II samples or simply a reduction in the percent composition of the polysaccharide moiety of the LPS, it is very probable that the LPS II is merely the LPS which was fragmented or truncated in the isolation procedure. This is further supported by the fact that the LPS I portion of the LPS may either comprise the greater portion of the total LPS, as shown by the column chromatography profile, or the lesser portion of the total LPS and produce the same results upon analysis (data from previous work <9> and subsequent work <10 and 16>, as well

as work for this paper). It would seem that the conditions under which the bacteria are grown (8L bottle with forced filtered aeration or 1L flask on a shaker) or harvested (pelleting of bacteria during growth phase or rest phase, etc.) or (and?) the care taken during the LPS isolation process are more important in determining the relative quantities of LPS I and LPS II than is any intrinsic molecular difference between the two. It appears then that LPS I contains the complete LPS and LPS II contains fragments.

In the analysis of figure 24 only three lanes will be considered: HC 205 LPS I (lane 5), USDA 205 LPS I (lane 1), and USDA 205 LPS II (IND) (lane 4). Lane 4 is used rather than lane 3 simply because it stained darker.

Band "A" is present only in HC 205 LPS I and USDA 205 LPS I. This high molecular weight band is completely absent in the induced USDA 205 LPS II. Band "B" is present in all, but appears to be much more heavily stained in HC 205 LPS I and USDA 205 LPS I than it is in the induced sample. Due to the relatively large quantity of material at band "B" and band "C" in HC 205 LPS I and USDA 205 LPS I, it is difficult to discern two separate bands. However, in USDA 205 LPS II (IND) there are two separate bands. Band "D" and band "E" appear to be present in all three samples, though it is difficult to distinquish the two in HC 205 LPS I.

The most interesting band is "F". Though present in all three, it is dramatically darker in the lane containing induced sample than in either of the others. This is in spite of the indication given by the bottom most band that both HC 205 LPS I and USDA 205 LPS I are more heavily stained overall, which suggests that they probably contain more sample material.

Band "G" is much darker in the non-induced samples than in the induced USDA 205.

Due to the smearing of the bands in this gel, it was rather difficult to draw any conclusions as to the nature of the samples. Clearly what was needed was a technique which prevents aggregation of the LPSs in order to obtain more resolution. This was achieved by using a different detergent, deoxycholic acid (DOC) in place of the SDS (20).

Figure 25 shows the results of a 16cm DOC-PAGE gel (see caption for lane identification). Comparison of this gel with the SDS gel described above shows that the highest molecular weight band, which is present only in the non-induced samples, actually consists of two bands located very close together. That area which was labeled band "B" in figure 24 is now shown to be two or possibly three separate bands, only one of which is heavily stained in USDA 205 LPS II (IND). What were labeled bands "C" and "D" in figure 24 are more easily discernable in the non-induced lanes in figure 25. Only one of these bands appears in USDA 205 LPS II (IND). This is followed by a band which is

present only in USDA 205 LPS I and LPS II. Interestingly it is more intense in the LPS II. Below this are two bands present only in the induced sample. Table 4 summarizes the results of the DOC gel for the USDA 205 LPS I, HC 205 LPS I, and USDA 205 (IND) LPS II samples. The band labels are from figure .

Lane 8 in figure 25 contains the LPS from cells which were grown in the presence of apigenin but with forced filtered aeration and not on a shaker. In previous experimentation this method was shown to produce limited induction (see page 23). The banding pattern of this sample appears to be an intermediate between that of the previously discussed induced and non-induced patterns.

Band	USDA205LPSI	HC205LPSI	USDA205(IN)LPSII
A	++	++	-
B	+	+	-
C	+++	+++	±
D	+++	+++	++
E	++	+	-
F	+++	++	++
G	+	-	-
H	±	-	++
I	-	±	++

TABLE 4. Summary of bands from DOC-PAGE (16cm) shown in figure . Symbols: +++, very intense; ++, intense; +, present; -, not present; ±, marginal.

A 32cm DOC-PAGE (13% acrylamide) was run to gain still more separation (figure not shown). It revealed that the those bands labelled "C" and "D" in figure 24 are actually five discrete bands separated by very small molecular weight differences.

The 32cm DOC-PAGE was followed by 15% acrylamide (16cm) DOC-PAGE which was subjected to pre-electrophoresis. The purpose of pre-electrophoresis is to eliminate residues from the gel matrix. Figures 26, 27, and 28 show photos taken of this gel at various degrees of destaining (destaining was done using Kodak rapid fixer). A drawing of the banding pattern was generated (figure 29) from these photos (as well as others not shown) and compared to the banding pattern from Salmonella minnesota. Each repeating unit of Salmonella minnesota LPS contains five sugar residues: two galactose residues, two N-acetyl-galactosamine residues and one N-acetyl-glucosamine residue (22). These repeating units have a molecular weight of ca. 934 (the water released in the formation of the glycoside bonds must be subtracted) and the distance of separation of each one on the gel has been termed one sal unit (or 1 sal). In figure 26 the lane containing USDA 205 LPS I shows that the first group of bands occurs approximately four to five sal units from the bottom of the gel, with the major bands almost exactly one sal unit apart (these are bands "B", "C", and "D" in figure 25). Eight to nine sal units above these bands (at 12-13 sal units from the bottom) is another group of bands in

which the major bands are also one sal unit apart (this group is band "A" in figure 25). Above the bands at 12-13 sal units there are two more groups of bands, each separated by eight to nine sal units. These bands did not appear in any previous PAGE results and became evident in this gel only after very heavy staining. Within these clusters of major bands, each separated by one sal unit (M.W.=934), a series of less intense bands separated by very small increments are present. These are most pronounced between four and six sal units from the bottom (see lane 7 <USDA 205 LPS I> in figure 27 and 28, and lane 1 in the drawing in figure 29), but are also perceptible between other major bands. There appear to be five minor bands which are associated with each major band in this region. This is significant because each repeating unit of Salmonella minnesota LPS is comprised of five sugar residues as previously described. When it is taken into consideration that each of the major bands of Rhizobium fredii USDA 205 LPS I (non-induced) migrate co-ordinately to those of S. minnesota, this banding pattern suggests that where the major bands from R. fredii may represent an oligosaccharide of approximately the same molecular weight as the repeating unit of S. minnesota, the minor bands may represent variations of one sugar residue.

The significance of the four to five sal unit spacing between the lowest band and the first cluster of bands and the eight to nine sal unit spacing between the subsequent

clusters of major bands is not known, although it is not uncommon in biological systems for a biosynthetic product to be assembled in one location and translocated to another when a certain criterion (or set of criteria) is met, such as chain length. What is very significant in relation to this spacing of the clusters is that the lane containing R. fredii USDA 205 LPS II (IND) is totally devoid of any bands past the group of associated bands at the fifth sal unit. This held true even when the gel was allowed to develop a very dark stain for even the most minor of bands. Of equal significance is the fact that the lane which contains LPS from R. fredii USDA 205 which was grown under conditions which allowed only limited induction (lane 5 in figures 26 and 27) shows a stepwise reduction in the molecular weights of those bands most intensely stained. There were no bands of higher molecular weight than eleven or twelve sal units. Unlike the non-induced LPS, the partially induced sample produced bands between five and twelve sal units. As in other gels the banding pattern of the partially induced sample appeared as an intermediate between that of the induced and that of the non-induced. In both the partially induced and induced samples there were bands at 1, 2, and 3 sal units which were either not present or barely perceptible (at 3 sal units) in the non-induced lane.

In the region of banding at four to six sal units from the bottom in the lane containing USDA 205 LPS I (non-induced), there is a difference in the spacing of the major

bands in relation to the minor bands with which they are associated. The major band at 5 sal units from the bottom (which is the most intensely stained band for this sample in all PAGE experiments performed) is associated with five minor bands, all located above it. However the major band at 4 sal units (the second most intensely stained band for this sample in all gels), also associated with four or five minor bands, is located not at the bottom of the group but one or two bands above the bottom (see figure 28, lane 7; and lane 1 in the drawing, figure 29). Although some of the minor bands associated with other major bands are visible in this gel, it is not possible to determine if the arrangement is similar to that of the group at 5 sal units, the group at 4 sal units, or if they have an entirely different arrangement. The location of the major bands within these groups would appear to be of great importance because it implies that there is an addition or deletion of a sugar residue or possibly a modification of a sugar residue at the terminal end of a long polysaccharide chain. Since the most intensely stained bands represent predominant molecular species of specific molecular weights, these additions, deletions or modifications of terminal sugars may play a role in the promotion or termination of polysaccharide chain extension.

When the two major bands at 4 and 5 sal units in the lane containing USDA 205 (non-induced) LPS I are closely compared to the corresponding bands in the lane containing

USDA 205 LPS II (IND), it is evident that these bands are not perfectly adjacent to one another (figures 28 and 29). In addition, the vertical distance which separates them is less than the distance which separates the minor bands from one another and from the major bands in USDA 205 LPS I. This indicates that the difference in structure (and consequently the difference in molecular weight) between the corresponding bands in USDA 205 (non-induced) and USDA 205 (induced) is not simply the result of the addition or deletion of a single sugar residue but the consequence of either a modification of a sugar residue (or residues) or a compositional modification of an entire component oligosaccharide. The modification appears to be the result of the induction of genes involved in the symbiotic process and also appears to be associated with the lack of higher molecular weight molecules in the induced sample. This theory is supported by the intermediate nature of the partially induced sample (lane 8, figure 27).

These results may help to explain the lack of a shoulder in the column chromatography profile for the induced sample (see figure 10). Since the average length of the polysaccharide moiety is markedly reduced in the LPS from the induced cells, the sample would be much less prone to fragmentation or truncation during the LPS isolation process. Therefore any fragmentation that does take place would be on a smaller scale and the difference in size between the whole LPS and the truncated LPS would be much

less.

If the LPS is indeed made up of identical repeating units then there would be certain bonds, at regular intervals, which are more labile than others. This concept is supported by the fact that the percent composition of the LPS II samples, as determined by GC and colormetric analysis, remains reasonably homogeneous in spite of the fact that they appear to be fragments of whole LPS (see GC, MNR, and colormetric results, this section; a clarification of this point is in the discussion section of this paper). Integration of the NMR peaks (data not shown) has given additional support to the homogeneity of composition.

DISCUSSION

From the evidence presented in the results portion of this paper it is clear that the association of Rhizobium fredii with plant root material, or in this case apigenin, results in structural alterations in the lipopolysaccharide portion of the cell wall. The most compelling evidence is from the PAGE results. Although a comparison of HC 205 and USDA 205 was the original intent of this research, it is difficult to ascertain what the differences might be because the gel pattern of HC 205 is much less revealing than those of the USDA 205 and the USDA 205 (IND). This in itself may be significant. What is needed is HC 205 LPS samples from induced and non-induced cultures which have been grown under the same conditions as the other samples. In this work the HC 205 was grown in an 8L bottle while the others were grown in 1L flasks on a shaker. This occurred because the HC 205 LPS was isolated before the need for induction was known, and it was the induction process that required the use of the shaker. There are differences between the experimental results of HC 205 and USDA 205, but with different culture methods and harvesting methods these differences can not be given any weight (cultures grown in large bottles require much more time to reach the same O.D. as an identical culture grown in a 1L flask on a shaker, and the harvesting of the cells at the same O.D. does not mean that they will be harvested under the same physiological conditions). The

USDA 205 and USDA 205 (IND) on the other hand were carefully grown under exactly the same conditions (that portion most often used in these experiments). Consequently the differences noted in the course of this study between these two samples may be given much more significance in the final analysis than any differences seen in the HC 205 LPS.

Precisely what the differences might be can not yet be determined. There must be further research in the area of sample purification and analysis before a clear picture of the LPS can be elucidated. At this time we are in the process of subjecting LPS to mild acid hydrolysis followed by purification of the resultant oligosaccharides through an gel-filtration column. These oligosaccharides will yield much clearer NMR and GC results. However, given the data from this research, some conjecture as to the nature of the changes brought about by the induction of the nod genes is warranted.

The LPS of bacteria is commonly composed of three parts: 1) Lipid A contains the fatty acid component attached to a dimer of D-glucosamine (in Salmonella typhimurium) (22). Glycerol is more common in other systems. 2) The core region is attached to the lipid A. It is an oligosaccharide whose structure is conserved in some species of bacteria and is variable in others (22). The lipid A and oligosaccharide core make up the R core. 3) The O side chain or O-antigen is typically made up of repeating units of identical oligosaccharides as described

for Salmonella in the PAGE results section of this paper.

It is the second and third regions which are the focus of this discussion. However, without the structure, or at least the composition of the O side chain repeating units, it is difficult to distinguish between the portion of the data which is due to the O side chain and that which is due to the core region.

The results of the DOC-PAGE analysis show that there is indeed a repeating unit which comprises the O side chain of Rhizobium fredii. The separation of bands by increments which are nearly equal to those that separate the repeating units of Salmonella indicates that a unit of ca. 900-950 M.W. is the basic unit of the R. fredii USDA 205 (non-induced) O-antigen. In addition, the 8-9 sal unit spacing between clusters of bands indicates that unlike Salmonella there is another larger scale enzymatic force at work in the assembly of the LPS of R. fredii.

The difference between the migration points of the predominant bands (at 4 and 5 sal units) in USDA 205 and USDA 205 (IND) indicates that the molecular weights or structures of these molecules are different. The difference is small indeed but not insignificant. As stated any compositional difference between the two may be the reason for the lack of O-antigen extension in the induced sample.

The data gathered from colormetric analysis and the quantification of the G.C. results indicate something of the overall composition of the LPS. G.C. results show that

there is a small but consistant amount of mannose in all the LPS samples. It has been shown in previous study that the core regions of other Rhizobium species contain a mannose residue (6), and the relative percent of mannose in these data indicate that there is only one mannose residue in these LPS samples. This eliminates a repeating oligosaccharide as the location of the mannose and indicates that it is in the core. The data on the methyl-hexose is not as reliable as that for mannose because it was generated not by a standard run but by comparison to another sugar (GLC). However the results would indicate the presence of at least one if not two of these residues, again pointing to the core region as their location in the LPS. There appears to be some glucose in the sample, but from previous work it is known that a B-glucan (probably from the peptido-glycan) is a common contaminant of these preparations. If it is not a contaminant then it would also be located in the core. Galacturonic acid has been shown to be a common constituent of the core regions of other rhizobia (6) and appears by colormetric analysis to be present in the non-induced LPS II samples at levels which would indicate no more than one residue in the core region of the non-induced samples if it is present there at all. However, there is a decrease in the relative percent of uronate in the LPS II of both HC 205 and USDA 205 when compared to the LPS I data from these samples. This suggests that a very small amount of uronic acid is present in the O-antigen since the LPS II samples

are presumed to be truncated or incomplete LPS (see results section) which would contain a higher relative percent of core sugars. Based on the known molecular weights (from PAGE) of the LPS in these samples and using the proportions of lipid A, core, and O-antigen present in Salmonella minnesota (which are typical of most gram negative bacteria), the approximately 4% uronic acid in the LPS I of the non-induced sample works out to two residues of uronic acid per five repeating unit LPS, of which one would be located in the core. This implies the modification or substitution of one sugar in one repeating unit of an LPS "monomer" (that is the four to five repeating unit LPSs shown to be the predominant species for HC 205 and USDA 205 <non-induced> in the PAGE results). It may be in fact that the addition of a carboxylated sugar residue to the terminal position of the O-antigen is one factor that prevents the addition of another 4-5 repeating unit group. This theory is supported by the 5.1% uronate composition demonstrated by USDA 205 (IND) LPS II. The predominant bands in the induced sample are of lower molecular weight and the addition of a carboxylated residue to these shorter LPSs would show up as a higher percent of uronic acid. There is an anomolous result for the LPS I of the induced LPS which shows a lower percent than for the LPS II (the colormetric results for the USDA 205 (IND) LPS I show a lower percent composition for all sugars tested except the acetyl group assay).

Colormetric and NMR data indicate the presence of small

amounts of pyruvic acid (pyruval groups) in all samples. This small amount suggests that it is also located in the core. The colormetric results show that the induced sample (LPS II) contains approximately half as much pyruvate as do the non-induced samples. Possibly the core of the induced LPS is also modified in some way.

Data from colormetric, G.C., and NMR (with integration of peaks <data not shown>) studies all indicate the presence of large amounts of KDO or a closely related sugar, as well as moderately large amounts of galactose. More recent G.C. analyses (with quantification of KDO by a standard) have yielded the same results. G.C., colormetric, and NMR analyses all indicate that KDO and galactose comprise 60-75% of the total LPS weight in a ratio of 2-3:1 (KDO:galactose). This gives direct evidence that these two sugars are present in both the core and the O-antigen, and may in fact be the exclusive constituents of the repeating units (with the exception of a modified terminal residue as previously discussed). KDO has long been known to be present in the LPS of nearly all gram-negative bacteria (24), and its presence here along with galactose is not at all unusual. What is unusual is the G.C. data which show not only the presence of KDO when compared to the standard but also peaks very close to the KDO peak, a result which suggests that they are related compounds. To date there is no evidence to suggest whether these are in fact products of LPS synthesis or byproducts of the isolation procedure

employed. If they are byproducts of the isolation and analytical procedures used, then the fact that they only appear significant in the G.C. profiles from the non-induced samples would indicate a difference between these two. At this time LPS samples are being prepared for mass spec. analysis to determine the nature of these products.

The evidence gathered to date suggests that the major differences between the induced and non-induced LPSs lie in the overall size of the LPS units (smaller after induction) and either a modification of some sugar residues in the LPS or a modification of the arrangement of the residues after induction. There is also evidence to suggest that the addition of a carboxylated sugar residue (probably galacturonic acid) may terminate O-antigen chain growth. The PAGE results indicate that one "group" of 4-5 O-antigen repeating units is linked to the core region and that if there are suitable conditions (e.g. the lack of a carboxylated terminal sugar) additional O-antigen in the form of two 4-5 repeating unit "groups" linked together may be added to continue chain growth. After induction the suitable conditions for chain growth, as well as the conditions necessary for production of complete 4-5 repeating unit "groups", would be limited or eliminated.

This hypothesis is not out of line when it is taken into consideration that the bacterial cell wall is thought to play a role in host recognition of the cell and plant lectin binding of the cell prior to root hair curling and

nodulation (17). Possibly a plant root exudate would induce the modification of a free living "wild type" cell to produce a cell different in genetic activity and molecular morphology that is subsequently recognized and bound by the host plant roots. This is not to say that the bacterial cell has been mutated to produce a different species, but rather that it has been altered in such a radical way (particularly in genetic activity) as to produce an organism which is very different from the free-living antecedent. In addition it has long been noted that in the bacteroid state there is a reduction in the cell wall thickness of rhizobial cells (14). In fact work with R. leguminosarum has indicated the complete absence of the O-antigen (5). Presumably this reduction facilitates, amoung other things, the transport of usable (i.e., fixed) nitrogen out of the bacteroid and the influx of host-plant photosynthetate into the bacteroid.

This process of modification of the rhizobial cell to produce a physiologically and morphologically compatible symbiont is not at all unlike the process of cell differentiation and upon reflection appears to be a very unrefined and incomplete sibling of the process that is thought to have brought about the inclusion of a blue-green alga into a non-photosynthetic cell, resulting in the chloroplasts of higher plants.

In order to gain further data and clarify that which has already been gathered, several experiments have already

been initiated and others are in the planning stage.

As mentioned previously the LPS samples (ca. 30-40mg) have been subjected to mild acid hydrolysis (1% acetic acid, 100^{o}C for 1h), which hydrolizes the ketosidic bonds at the reducing end of KDO residues (these bonds are much more labile than the glycosidic bonds of other sugars and at other locations on the KDO molecule), followed by centrifugation to remove the lipid precipitate. This is followed by separation of the resultant oligosaccharides on a gel filtration (P2) column and collection of the fractions for NMR, G.C., and colormetric analyses. The lipid pellet is purified by chloroform-water extraction to remove any free polysaccharide that might have adhered to the pellet. This will be analyzed for fatty acid content and possibly the elucidation of the lipid A backbone and the first few core sugars if they are not KDO.

Further ELISA tests are being performed at this time to test the level of specificity between antibodies for induced and non-induced cells. If reasonable specificity is found the antisera will be employed in immunoblot assays of DOC-PAGE gels.

Samples of LPS are now being prepared for analysis by mass spec. with the goal of identifying those peaks from the G.C. analysis which have yet to be identified.

Other experiments concerned with structural analysis of the LPS include: the isolation of USDA cells from actual nodules to determine (by PAGE) if the O-antigen is

completely eliminated in the in vivo bacteroid; large scale isolation of HC 205 and induced HC 205 to compare to the USDA results and determine if the genes responsible for the structural modifications noted in this study are located on the plasmid which is missing in HC 205; and the methylation of oligosaccharides and whole LPSs followed by acetylation and G.C.-mass spec. analysis to determine the sequence of the O-antigen and core region.

Other work planned for the future includes the isolation of the enzymes responsible for the LPS modifications in the induced cells and ultimately the identification of the specific genes responsible for the production of these enzymes.

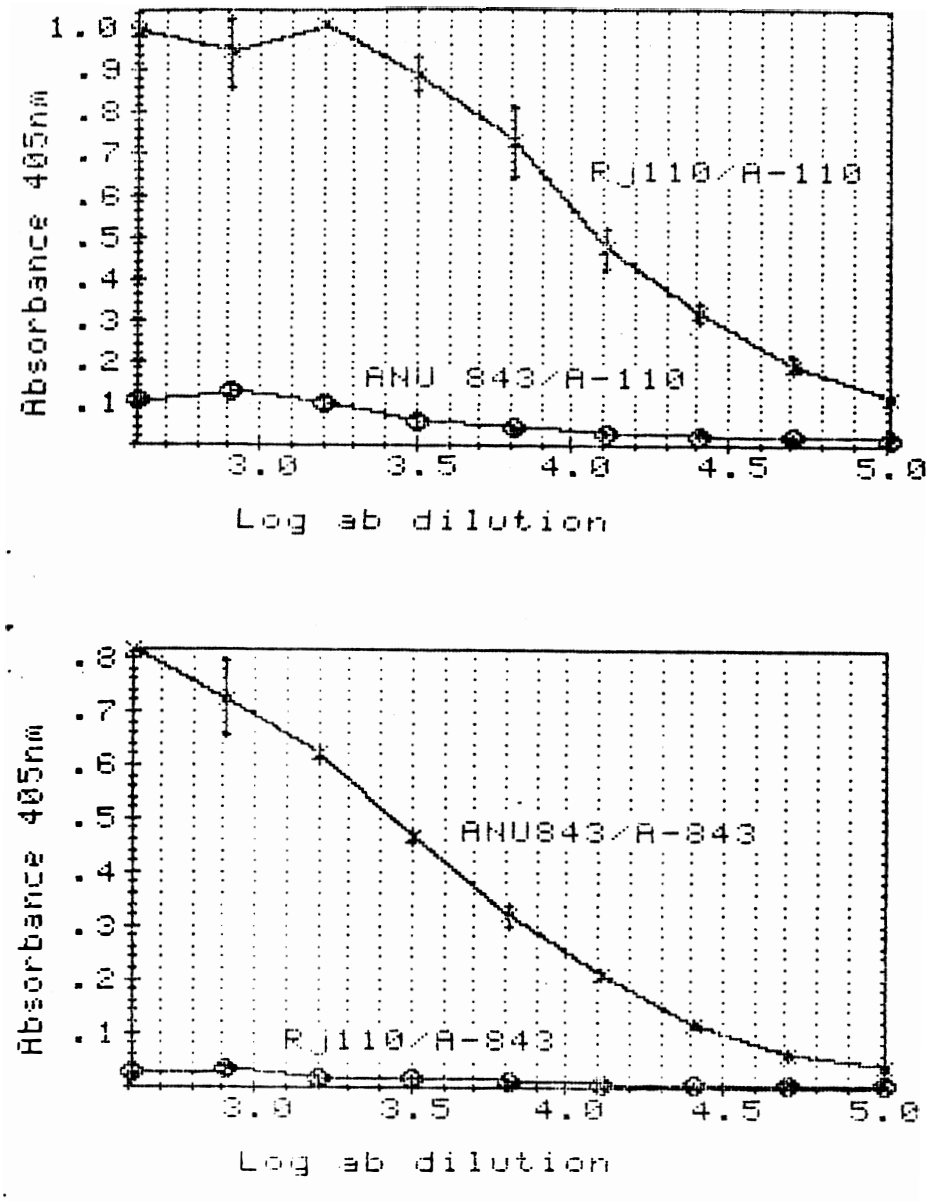

FIGURE 1. ELISA results using two different species of bacteria; Rhizobium japonicum USDA 110 (Rj110) and Rhizobium trifolii ANU 843 (ANU843), and antisera generated against them (<A-110> and <A-843> respectively). Upper graph, anti-110; lower graph, anti-843.

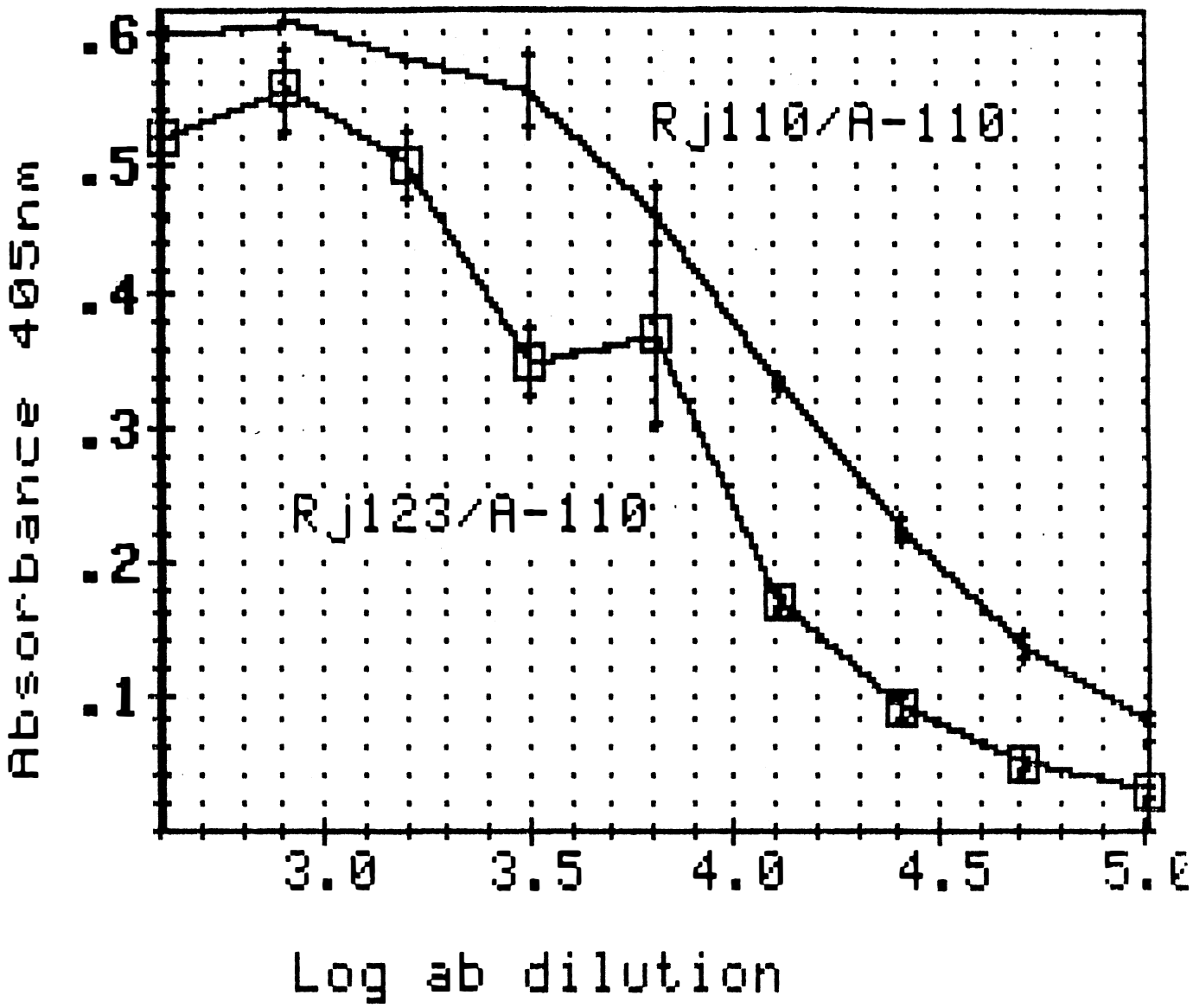

FIGURE 2. ELISA results using two different strains of the same species of bacteria; Rhizobium japonicum USDA 110 and R. japonicum USDA 123. The antisera is anti-USDA 110 (A-110).

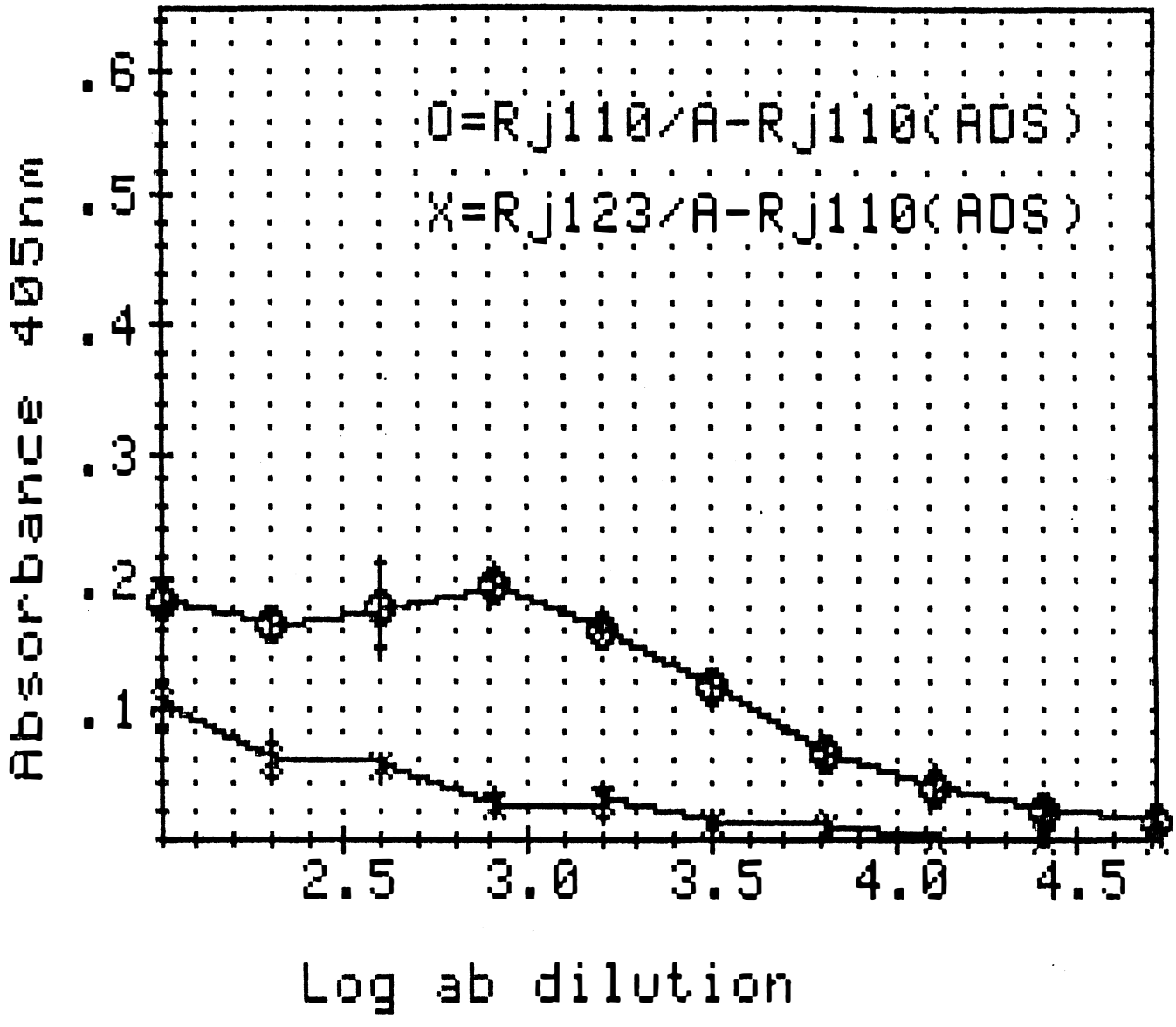

FIGURE 3. ELISA of two different strains of the same species, Rhizobium japonicum USDA 110 and USDA 123, after adsorption purification of the antisera (A-110 incubated with USDA 123 cells). Note the increased specificity accompanied by a reduction of overall absorbance when compared to Figure 2.

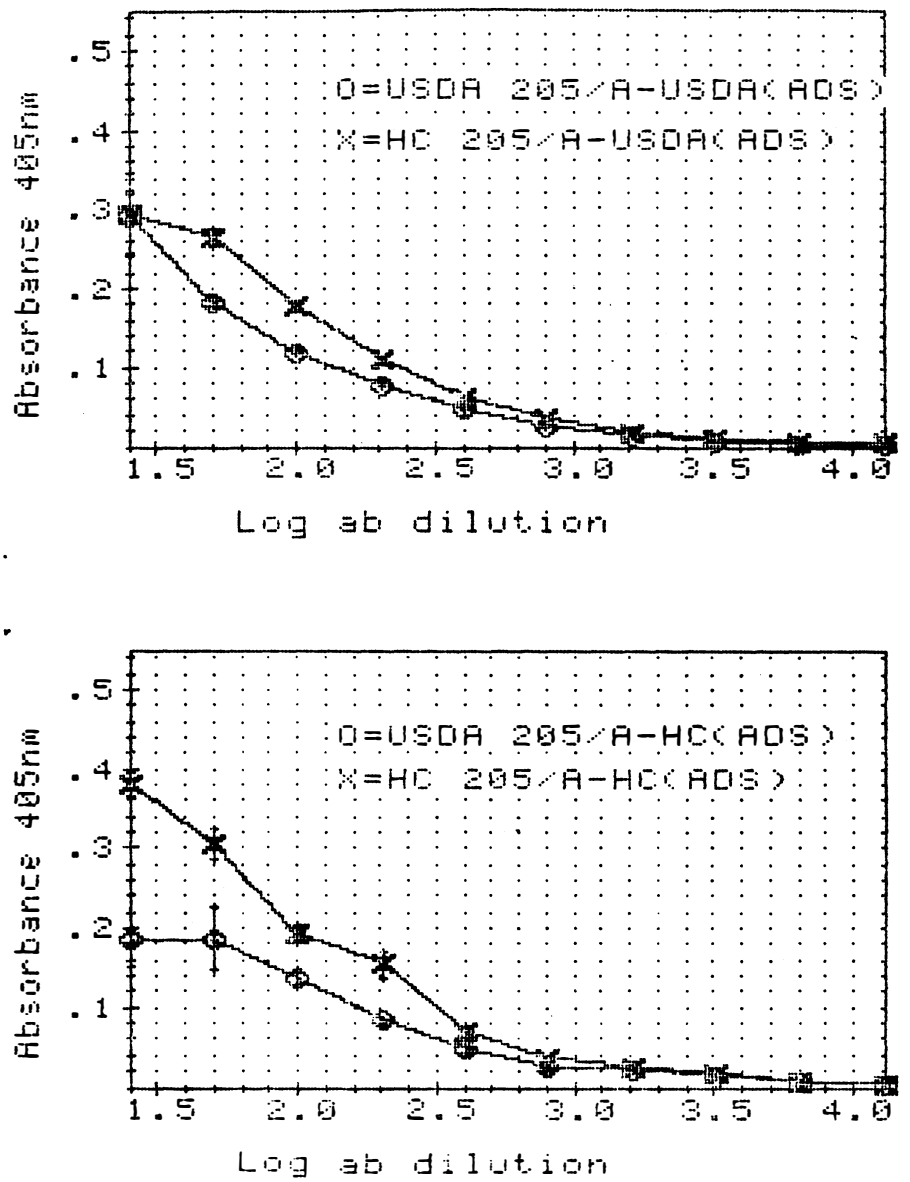

FIGURE 4. ELISA of Rhizobium fredii USDA 205 and R. fredii HC 205 using antisera which was purified by adsorption. Upper graph, anti-USDA (ads); lower graph, anti-HC (ads).

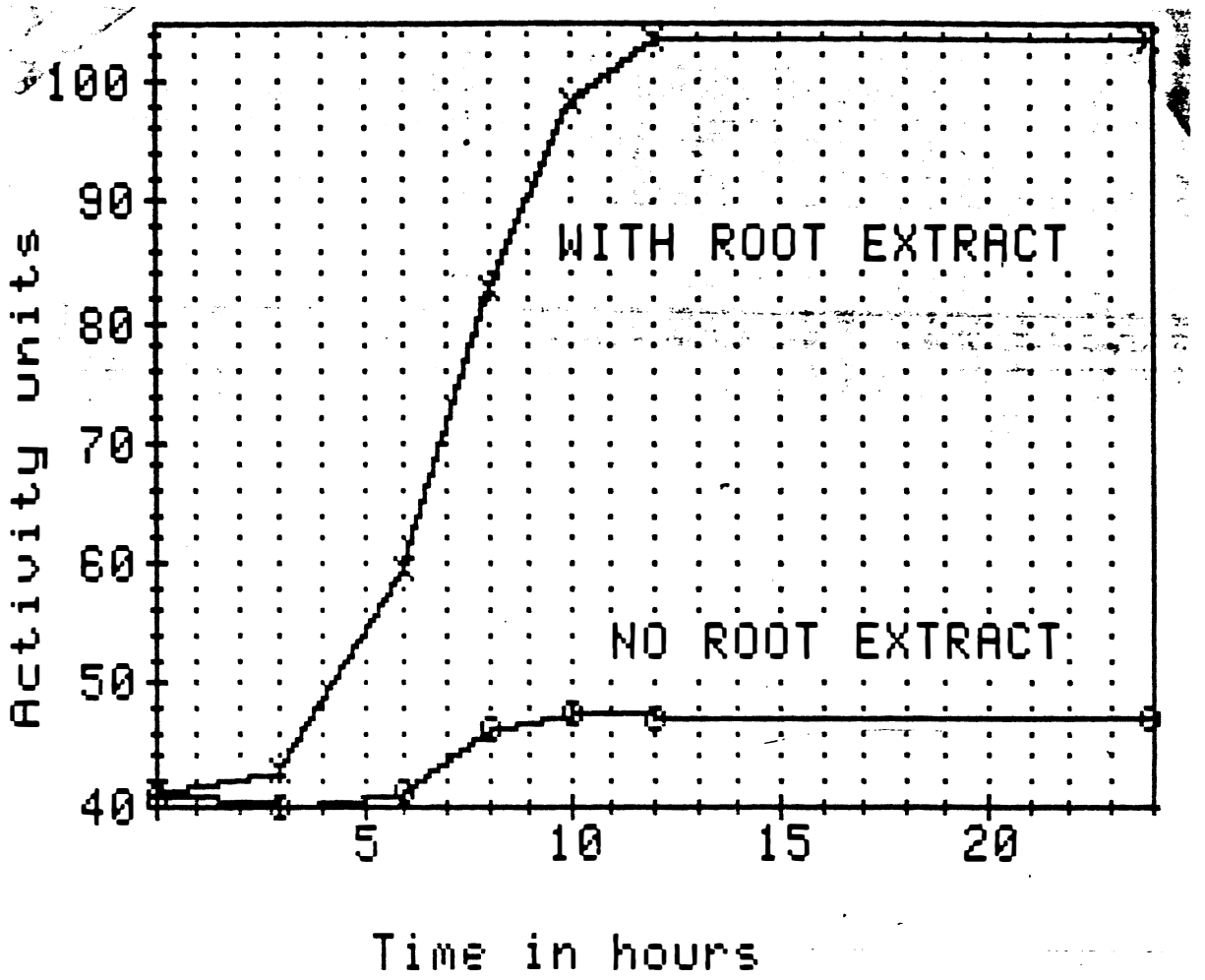

FIGURE 5. Induction of Rhizobium fredii USDA 201 Mu-lac mutant by association with soy bean root extract. See text for expanation.

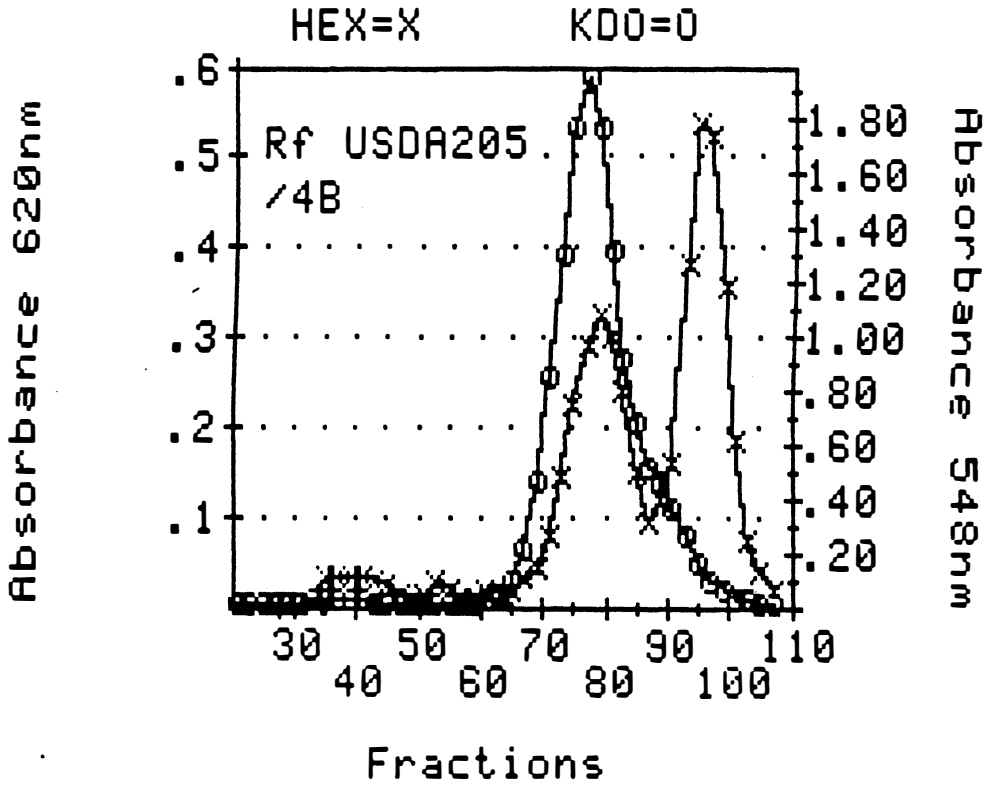

FIGURE 6. First 4B column chromatography profile of LPS from Rhizobium fredii USDA 205.

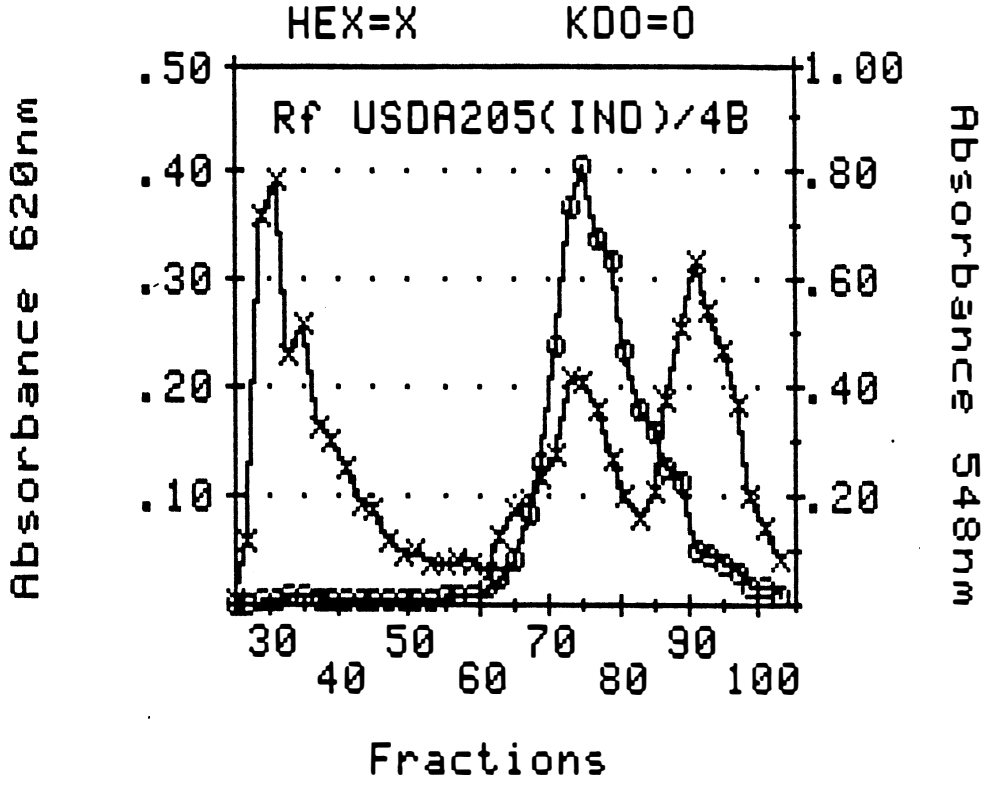

FIGURE 7. First 4B column chromatography profile of LPS from Rhizobium fredii USDA 205 (IND).

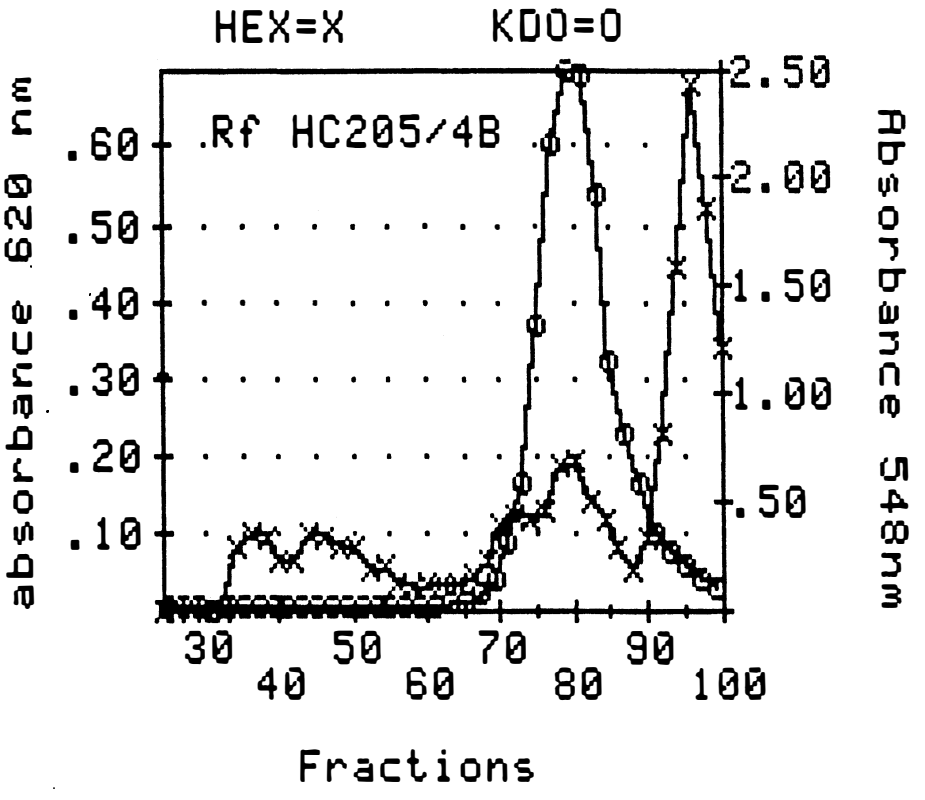

FIGURE 8. First 4B column chromatography profile of LPS from Rhizobium fredii HC 205.

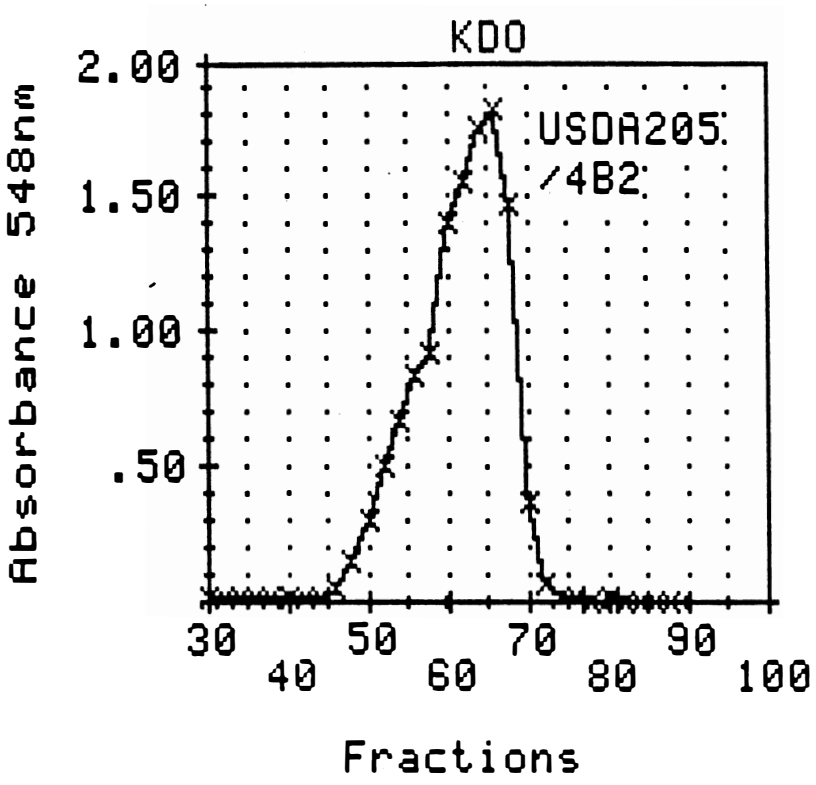

FIGURE 9. Second 4B column chromatography profile of LPS from Rhizobium fredii USDA 205.

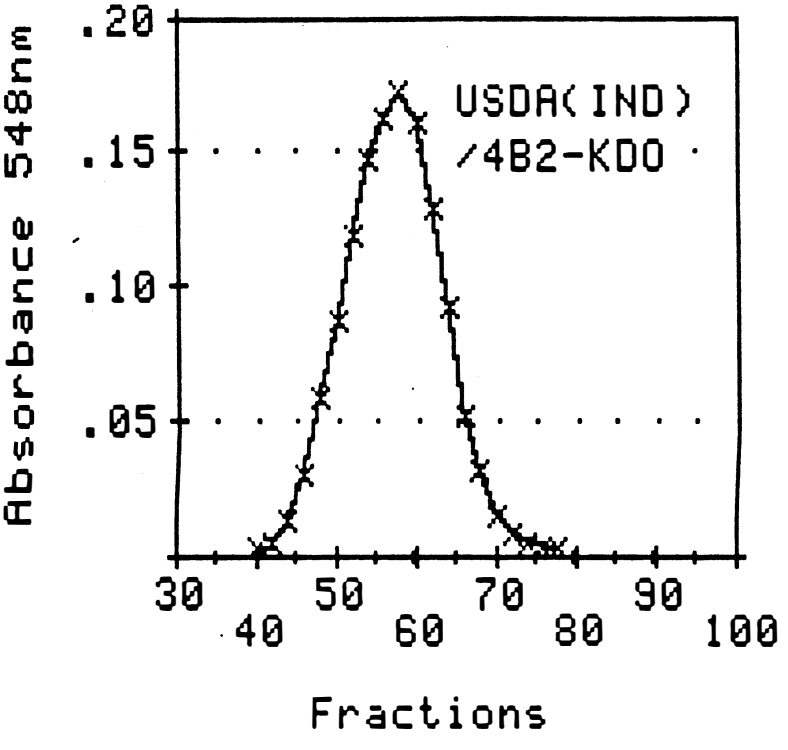

FIGURE 10. Second 4B column chromatography profile of LPS from Rhizobium fredii USDA 205 (IND).

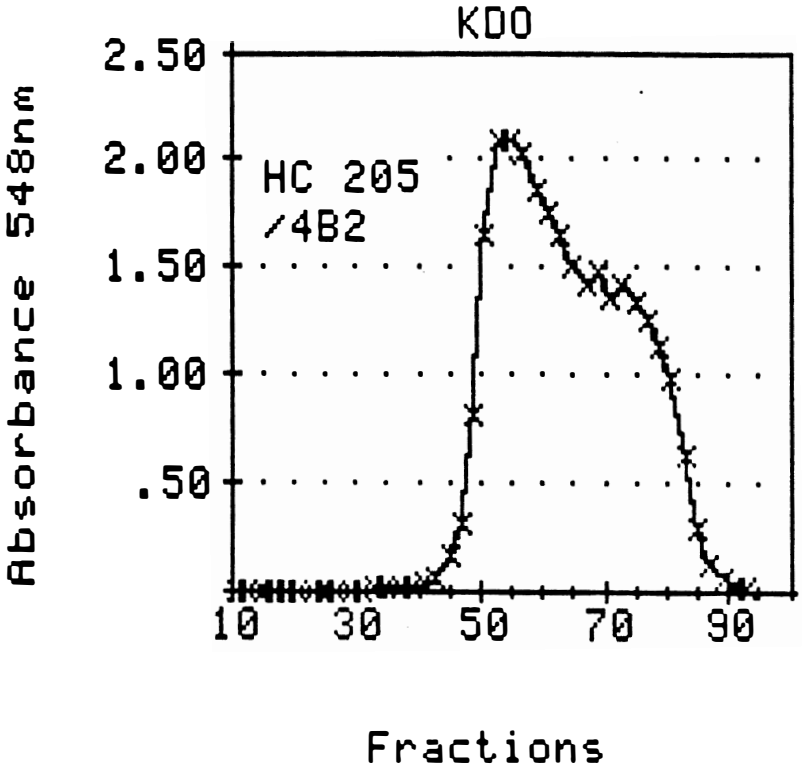

FIGURE 11. Second 4B column chromatography profile of LPS from Rhizobium fredii HC 205.

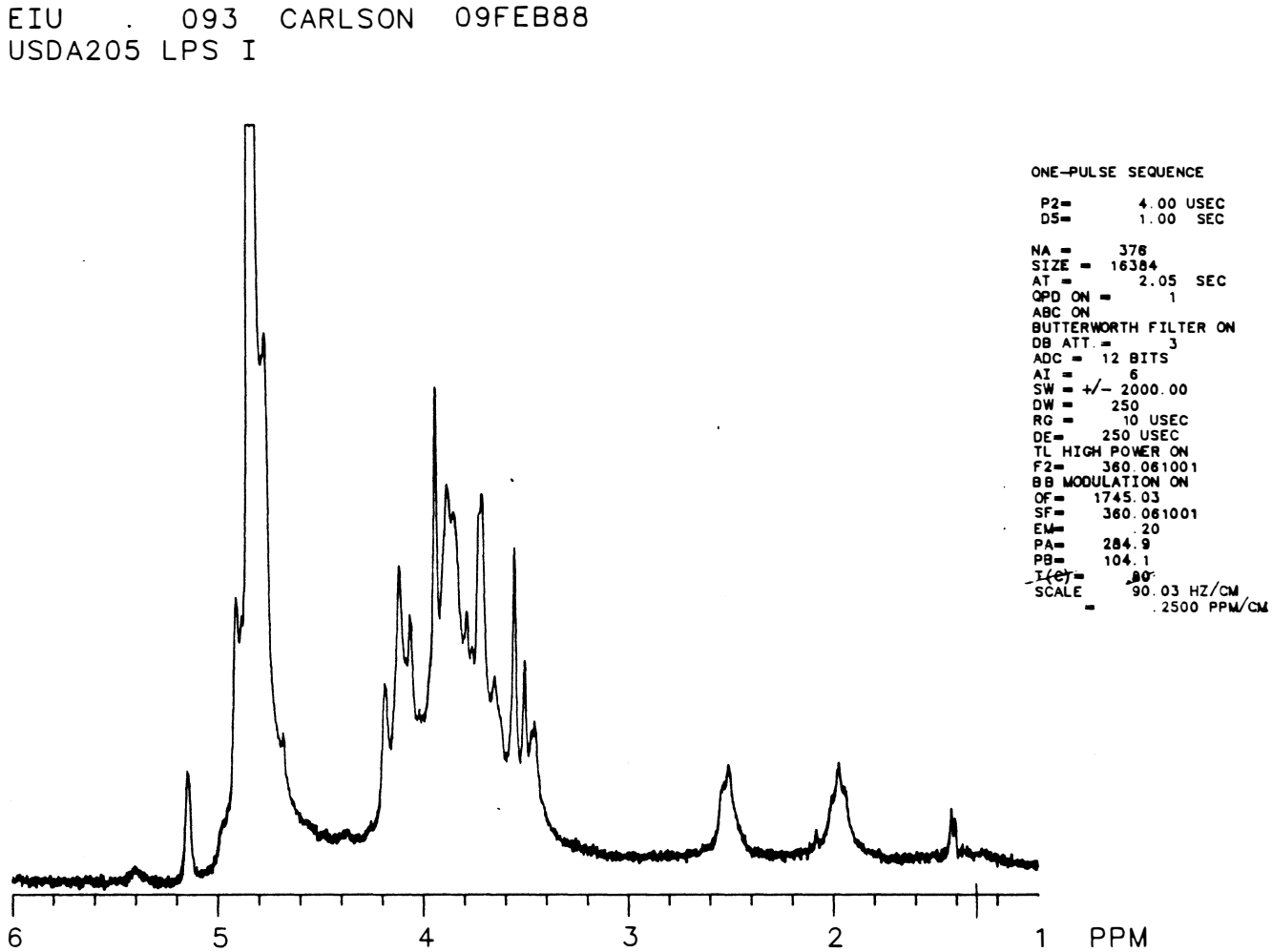

FIGURE 12. NMR spectra of Rhizobium fredii USDA 205 LPS I.

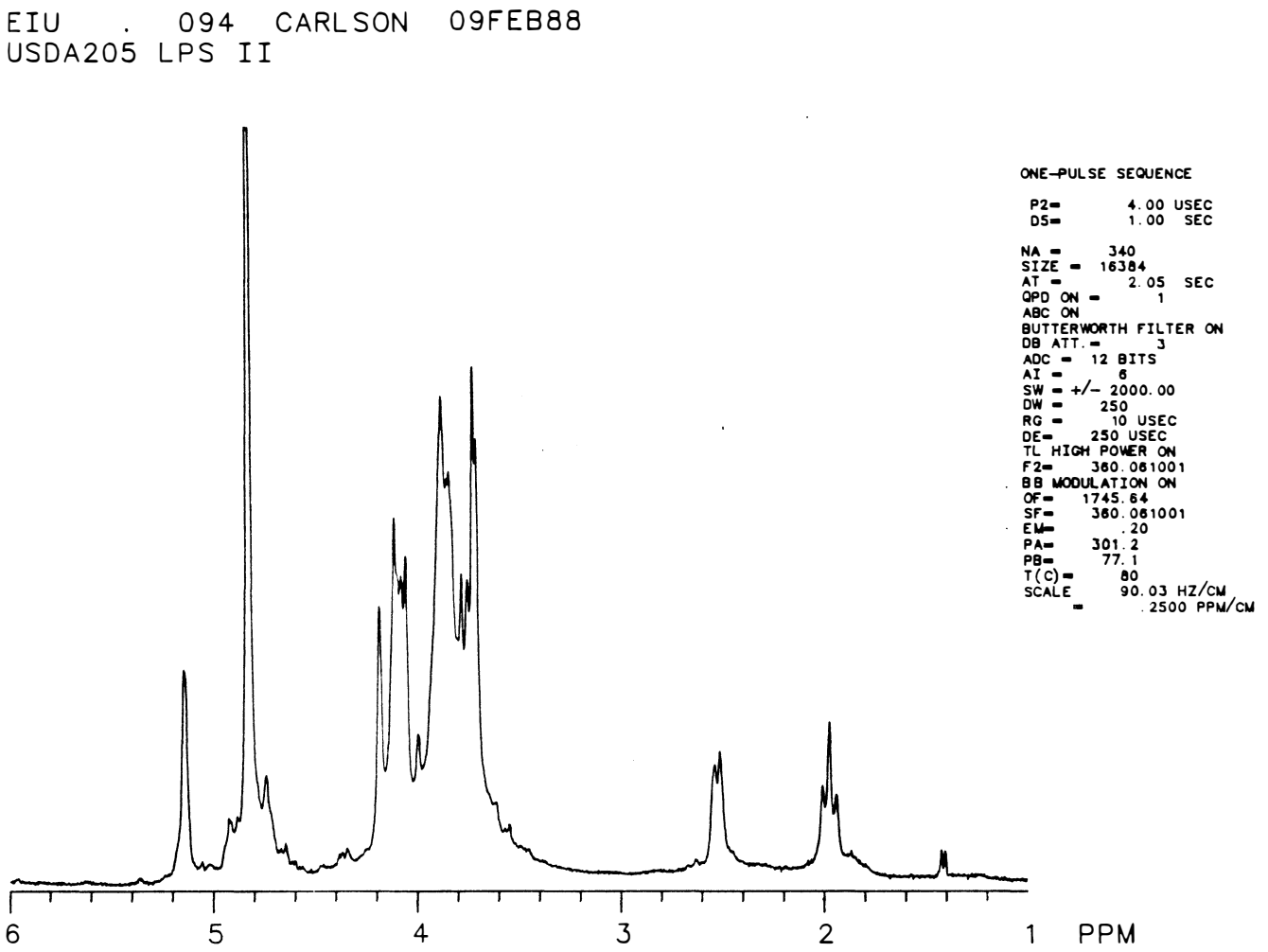

FIGURE 13. NMR spectra of <u>Rhizobium fredii</u> USDA 205 LPS II.

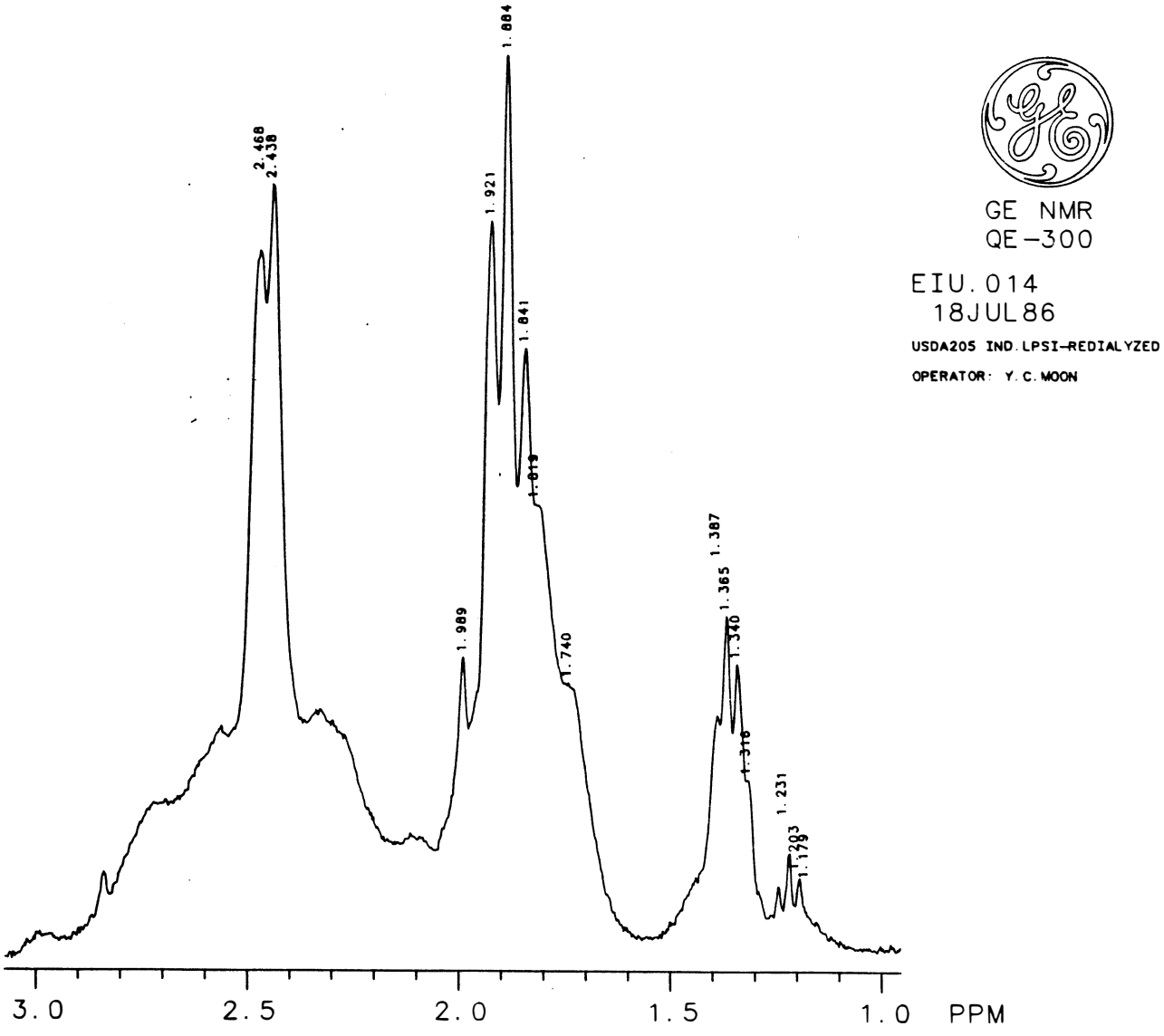

FIGURE 14. NMR spectra of Rhizobium fredii USDA 205 (IND) LPS I.

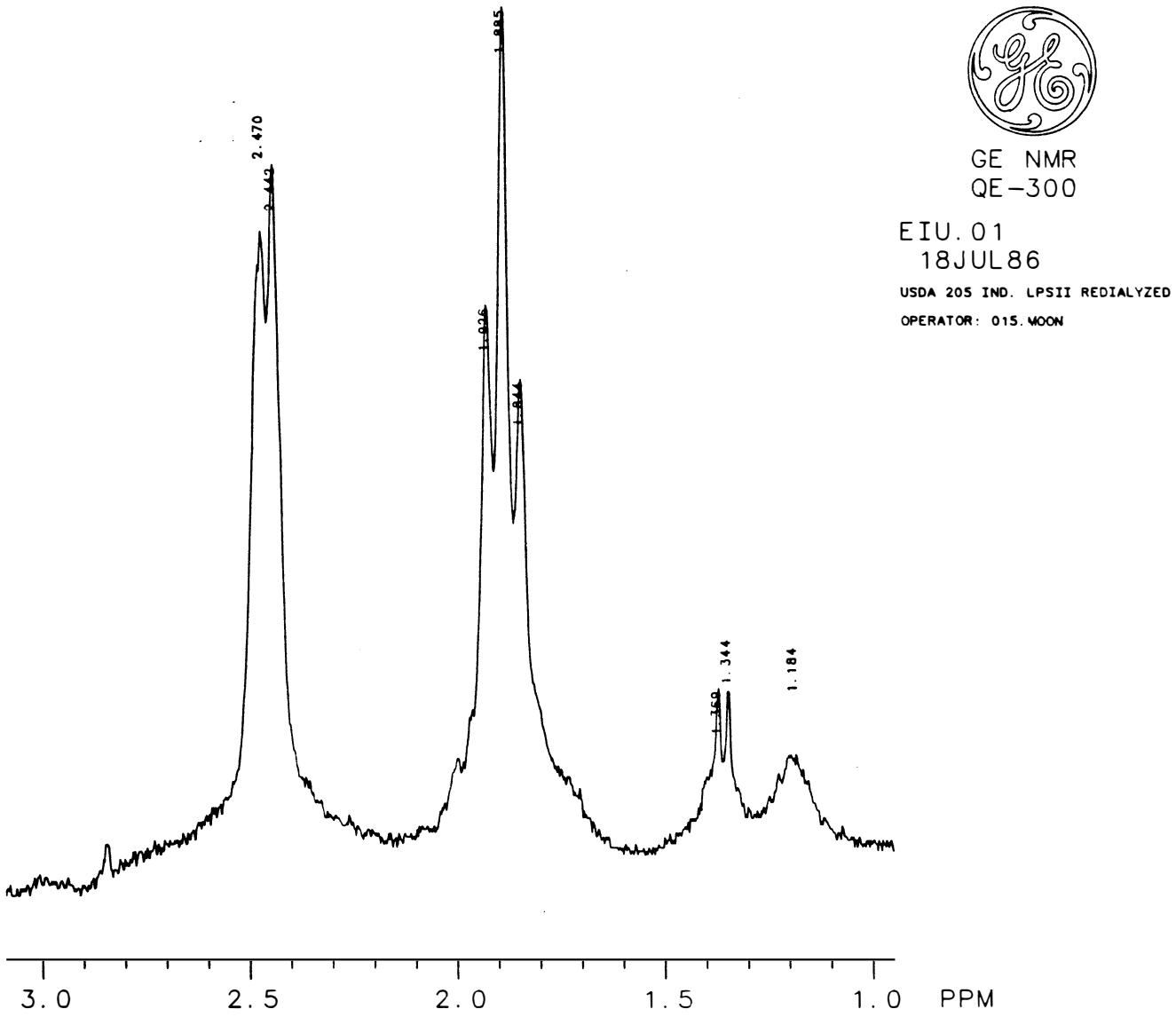

FIGURE 15. NMR spectra of Rhizobium fredii USDA 205 (IND) LPS II.

EIU . 096 CARLSON 09FEB88
HC205 LPS I

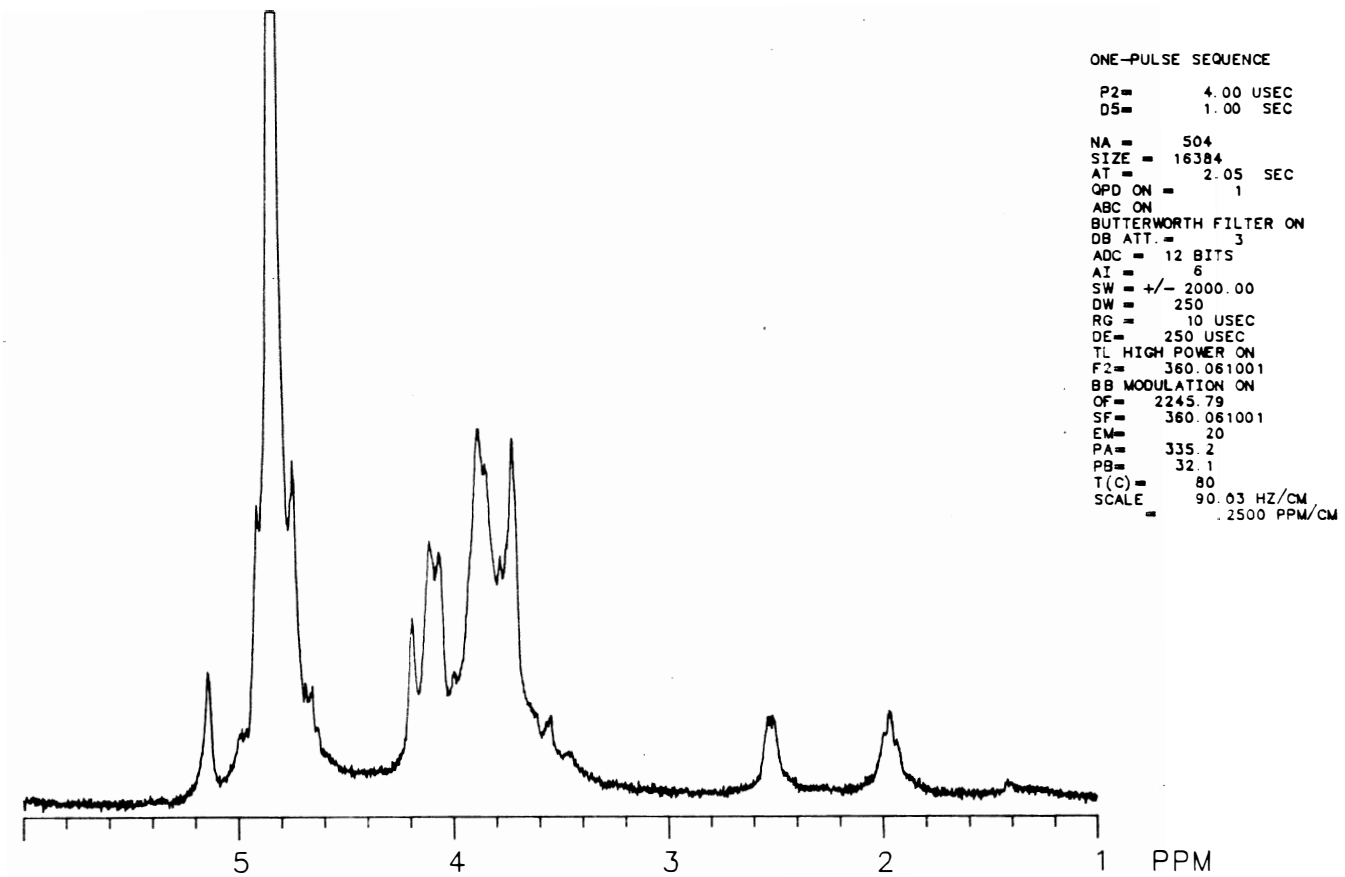

FIGURE 16. NMR spectra of Rhizobium fredii HC 205 LPS I.

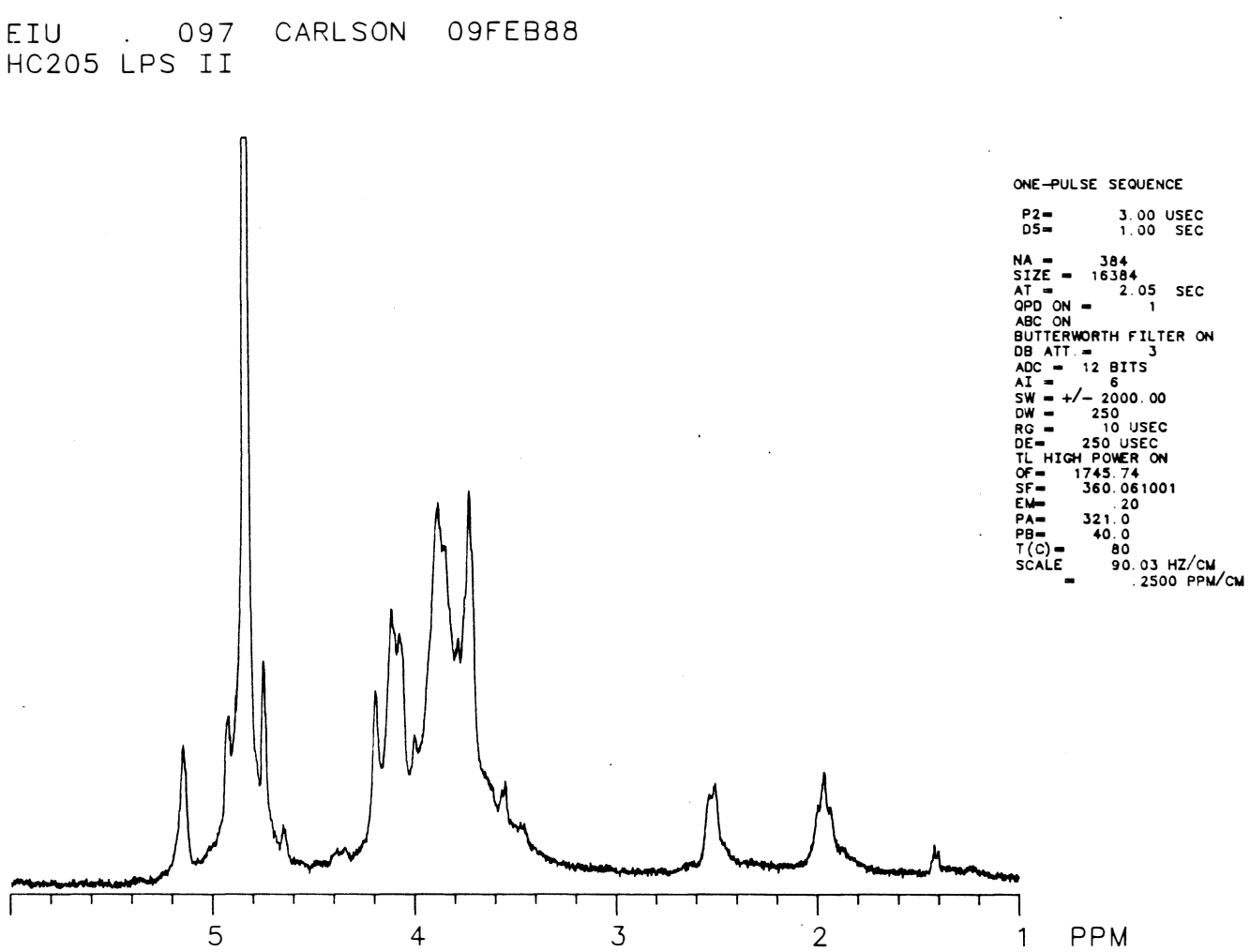

FIGURE 17. NMR spectra of Rhizobium fredii HC 205 LPS II.

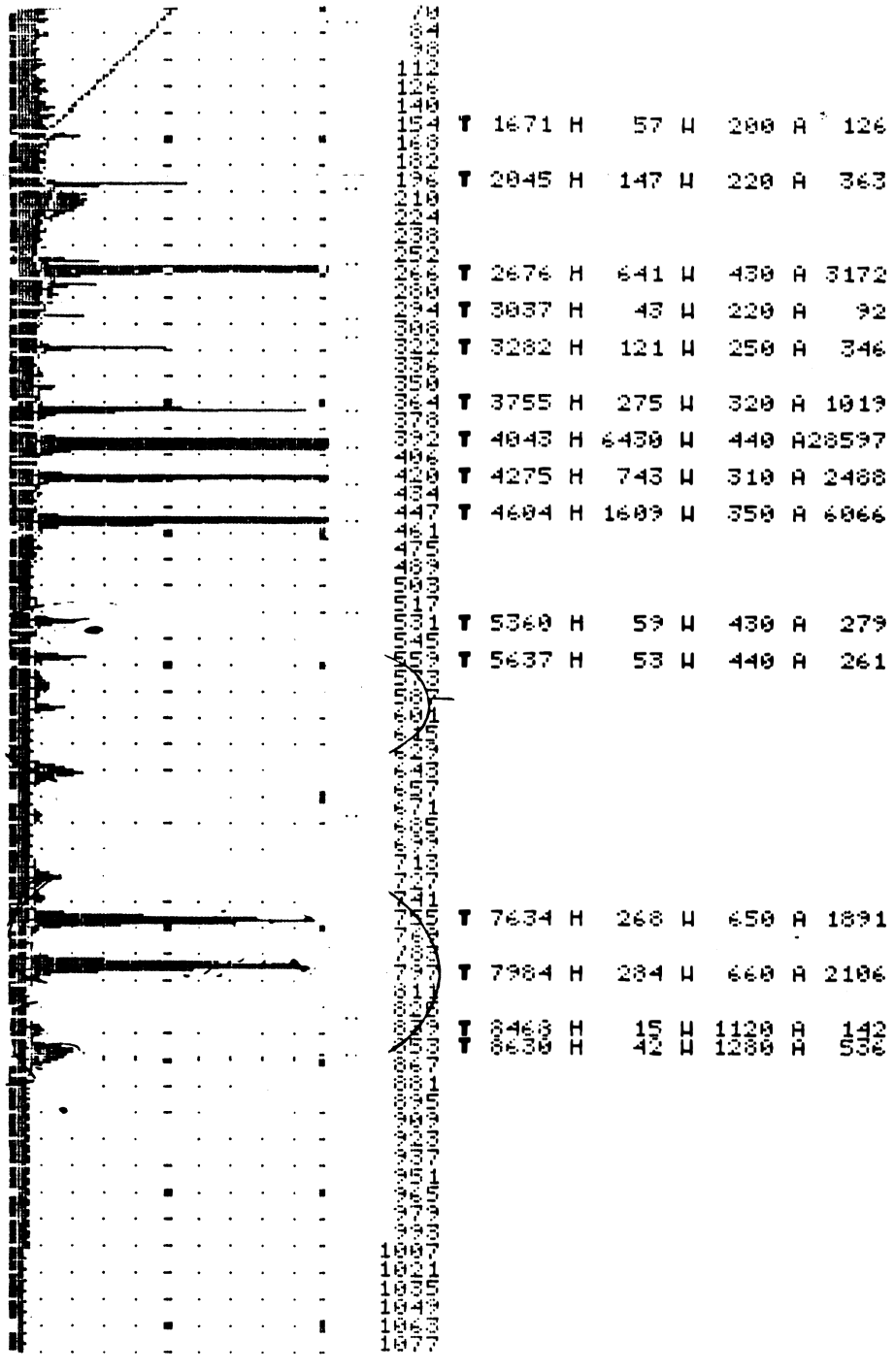

FIGURE 18. GC profile of Rhizobium fredii USDA 205 LPS I.

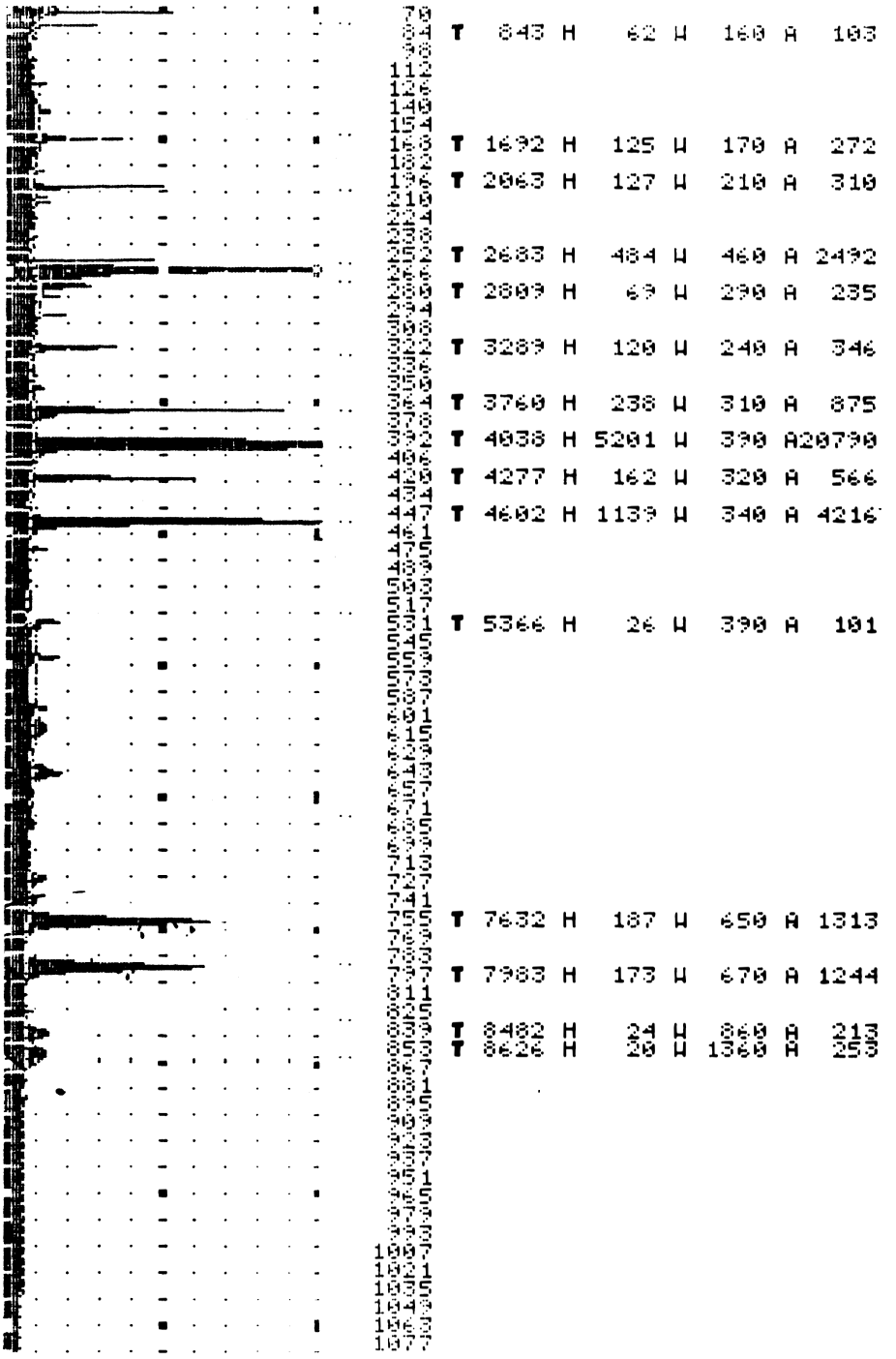

FIGURE 19. GC profile of Rhizobium fredii USDA 205 LPS II.

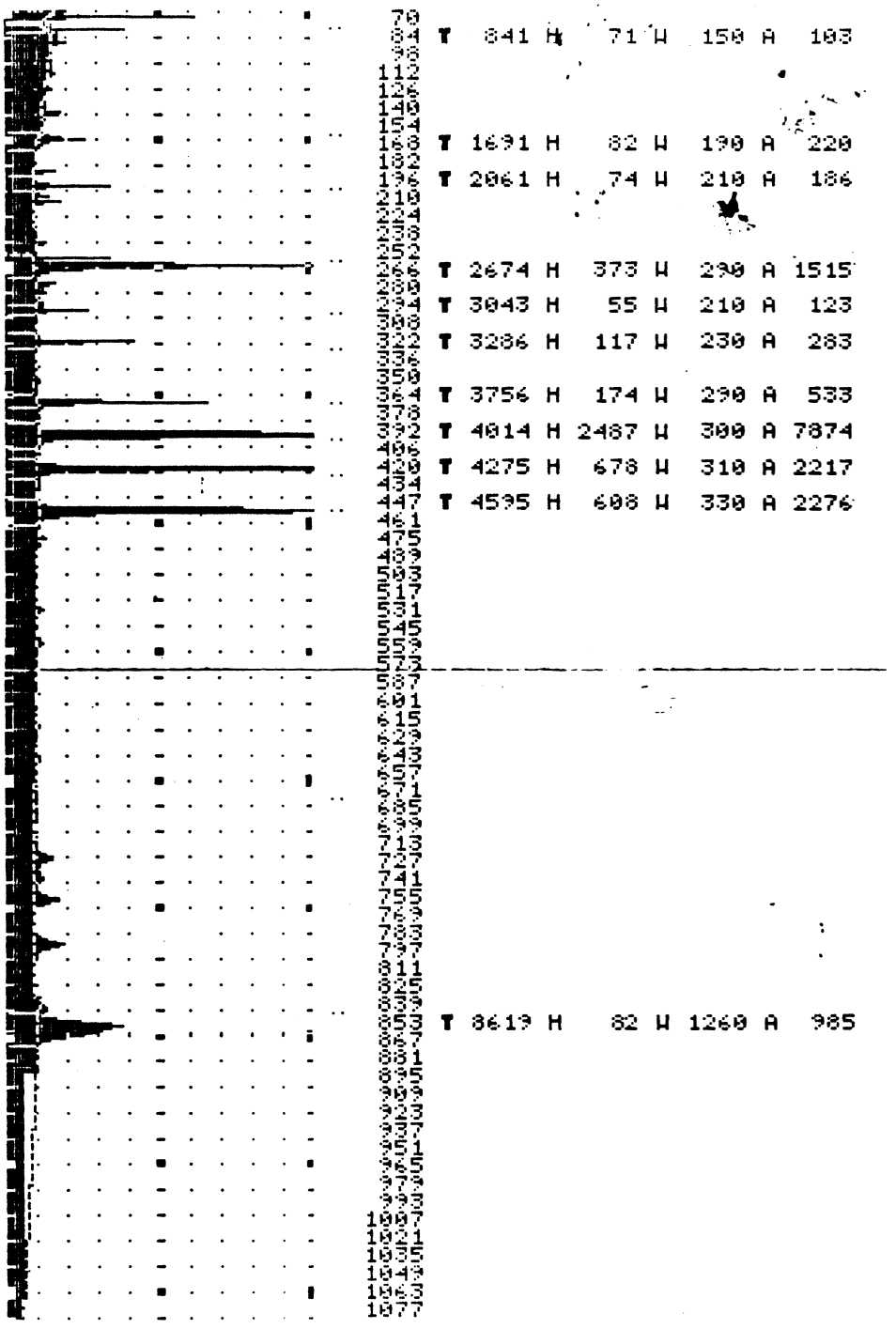

FIGURE 20. GC profile of Rhizobium fredii USDA 205 (IND) LPS I.

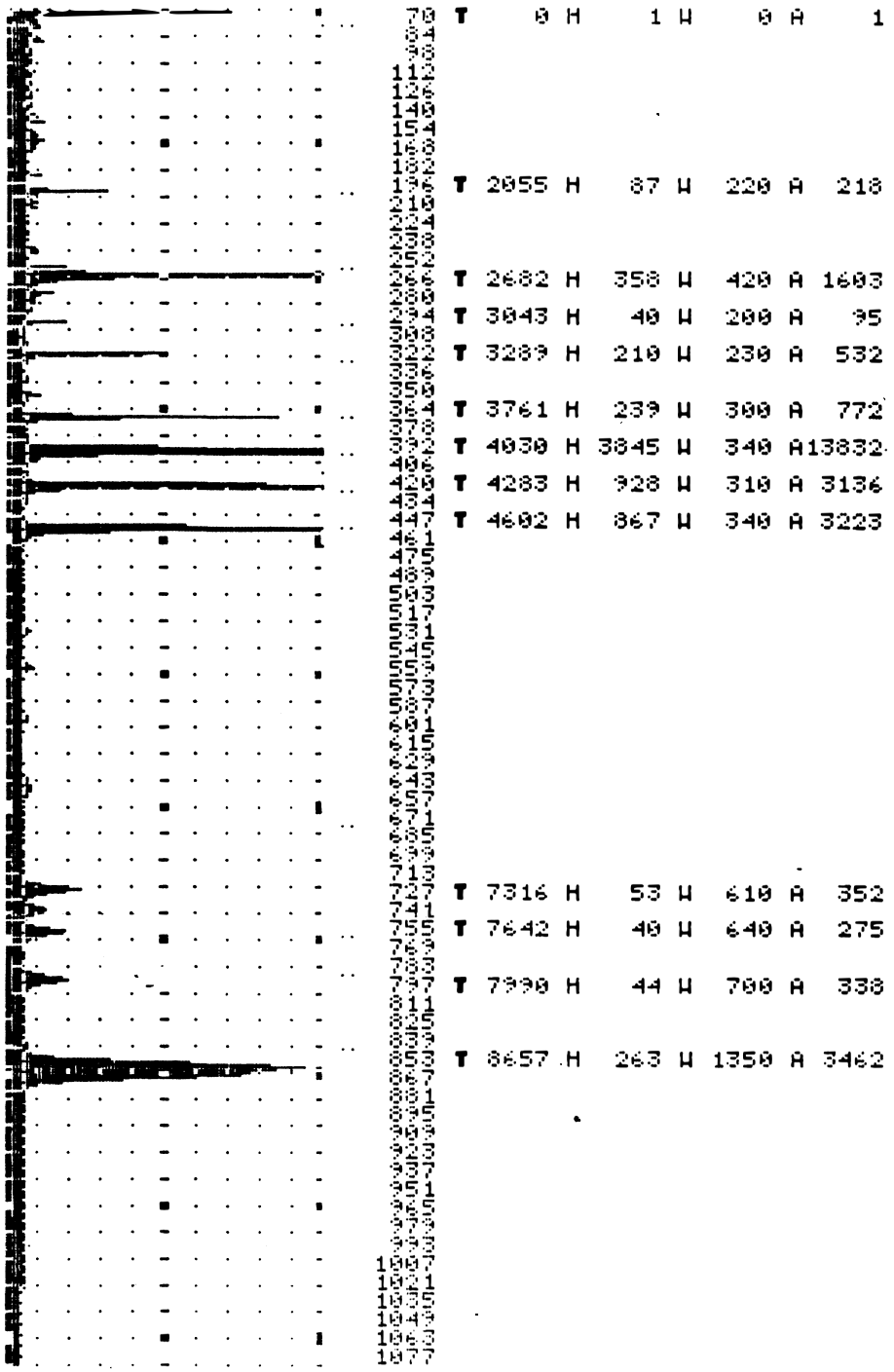

FIGURE 21. GC profile of Rhizobium fredii USDA 205 (IND) LPS II.

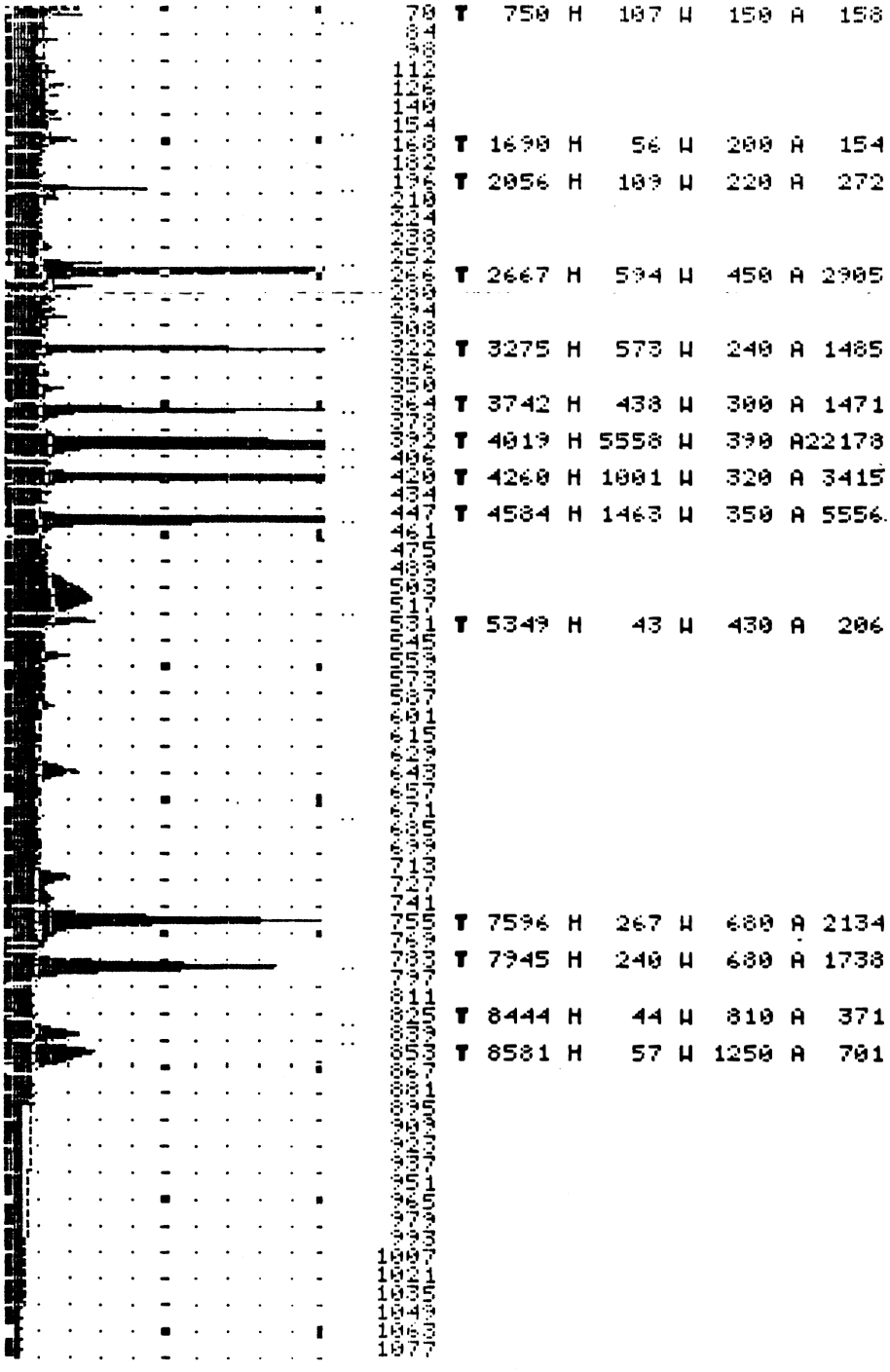

FIGURE 22. GC profile of Rhizobium fredii HC 205 LPS I.

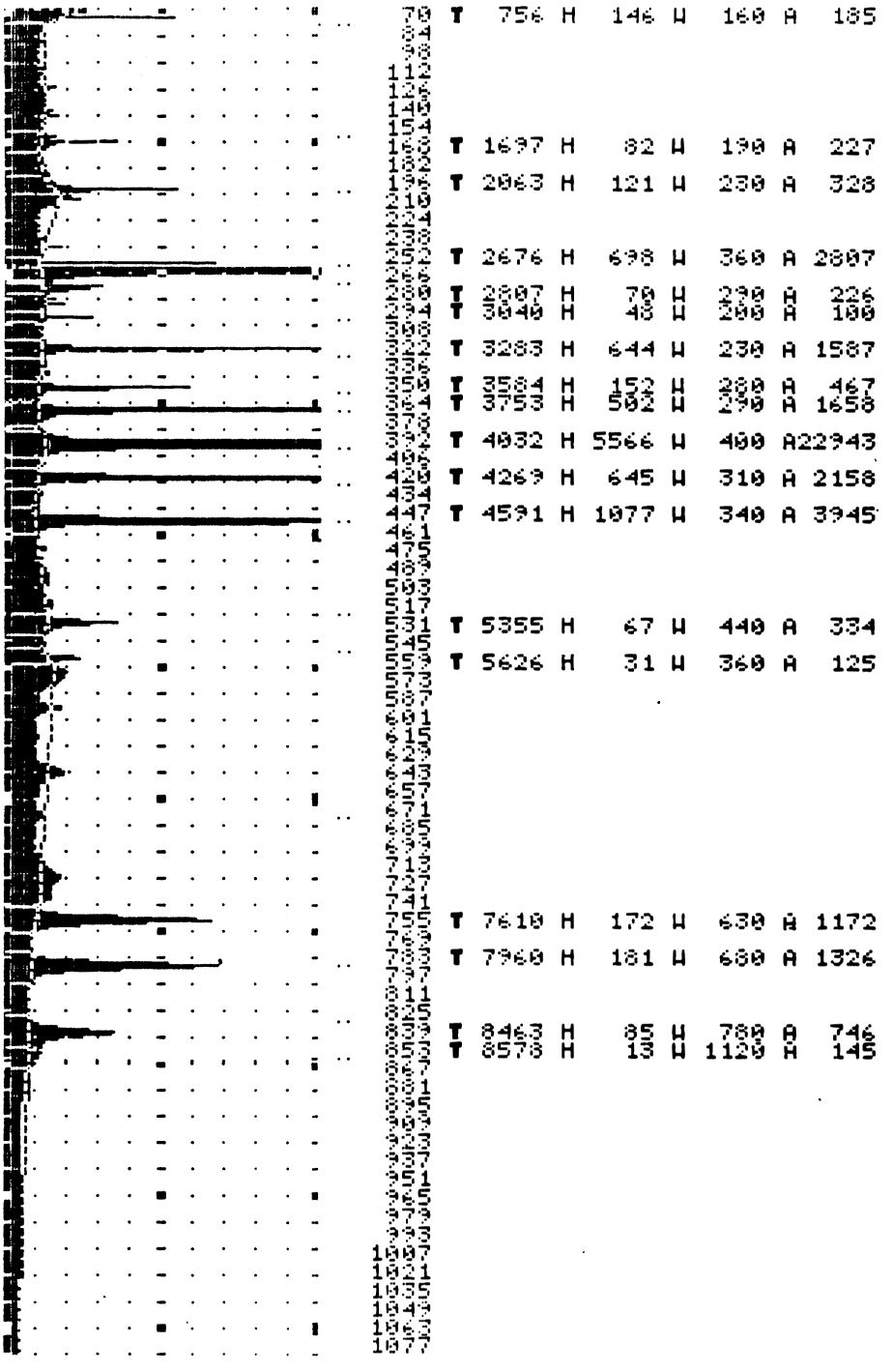

FIGURE 23. GC profile of Rhizobium fredii HC 205 LPS II.

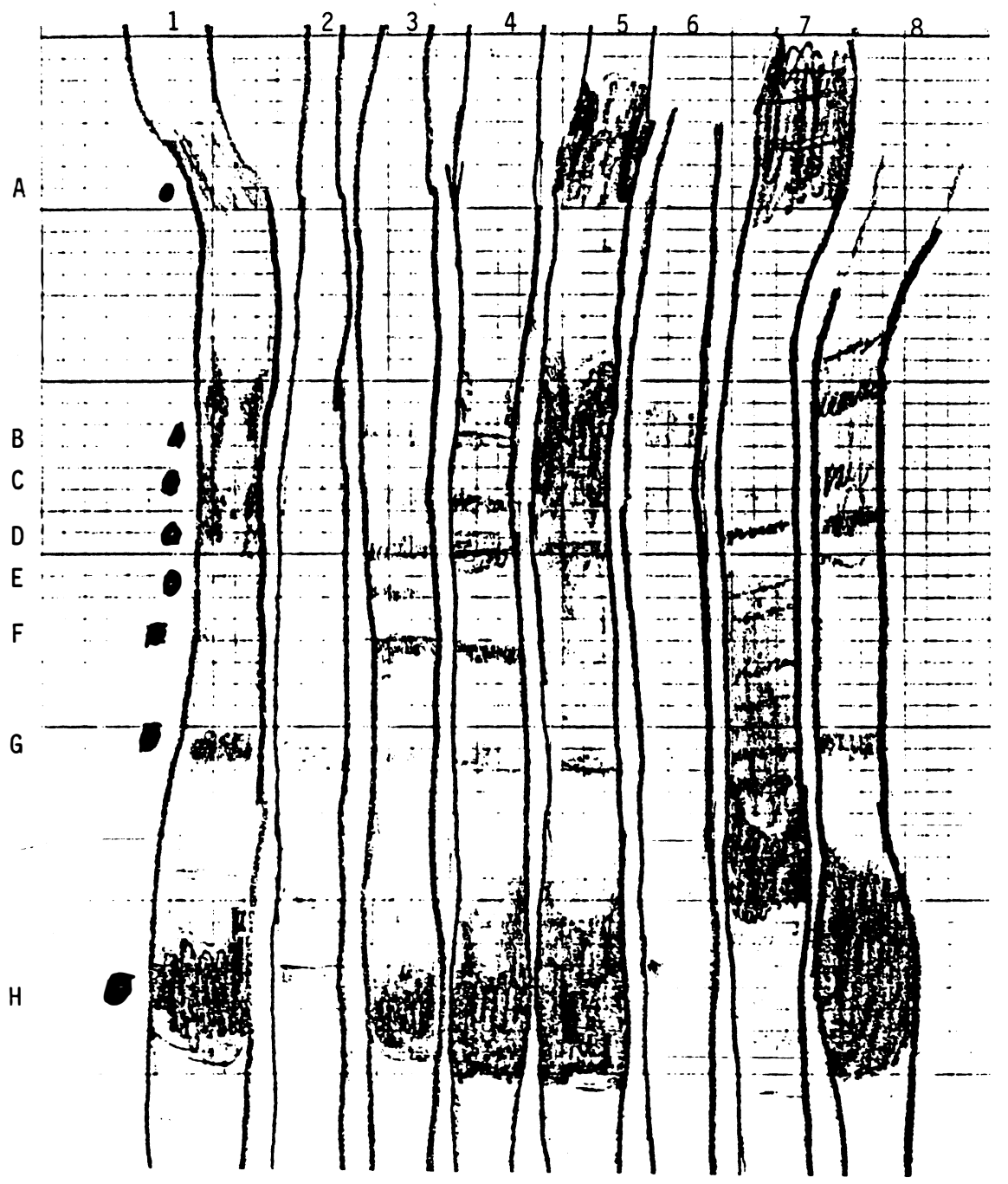

FIGURE 24. Drawing of SDS-PAGE gel. Lanes: 1) USDA LPS I; 2) USDA LPS II; 3) USDA (IND) LPS I; 4) USDA (IND) LPS II; 5) HC LPS I; 6) HC LPS II; 7) Standard, Salmonella LPS; 8) USDA (IND) LPS II, 2X sample. Letters to the left of figure represent band areas as discussed in text.

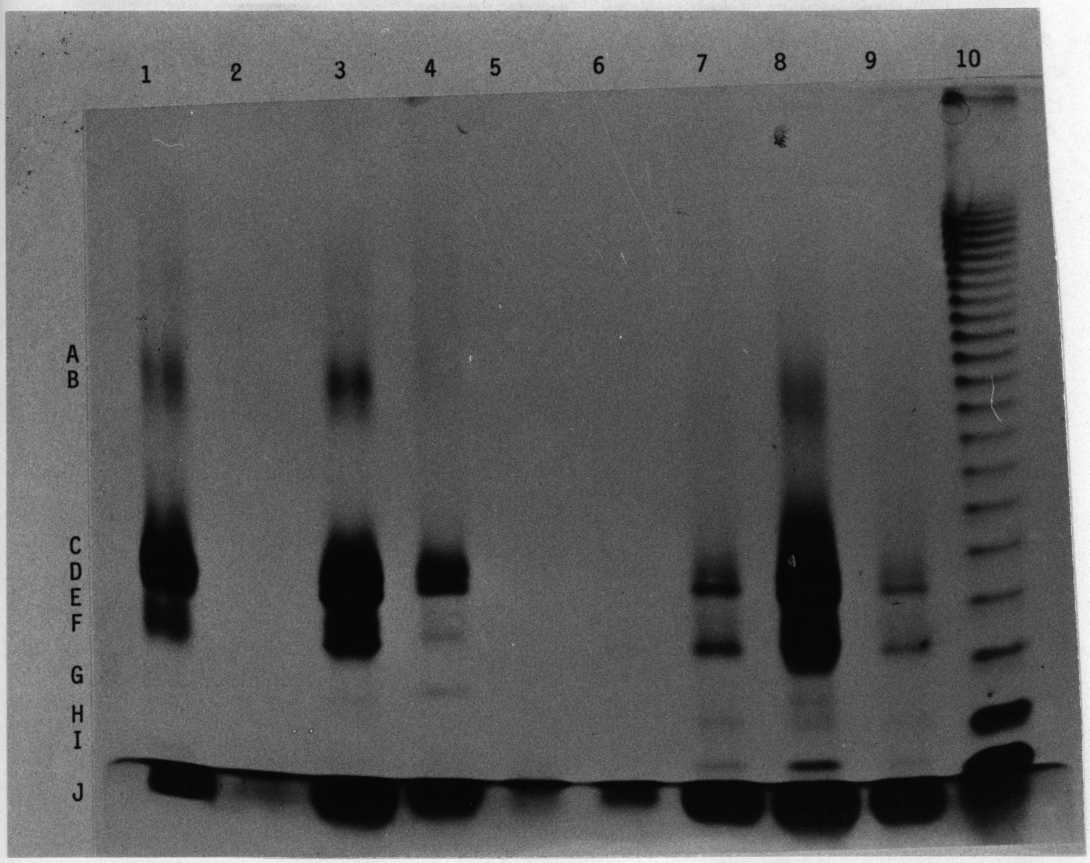

FIGURE 25. 16cm, 13% acrylamide DOC-PAGE gel. Lanes: 1) HC LPS I; 2) HC LPS II; 3) USDA LPS I; 4) USDA LPS II (from sample not used in this study); 5) USDA LPS II; 6) USDA (IND) LPS I; 7) USDA (IND) LPSII; 8) USDA (partially induced) LPS; 9) USDA (IND) LPS I+II; 10) Standard, Salmonella LPS. Letters refer to band areas (see text).

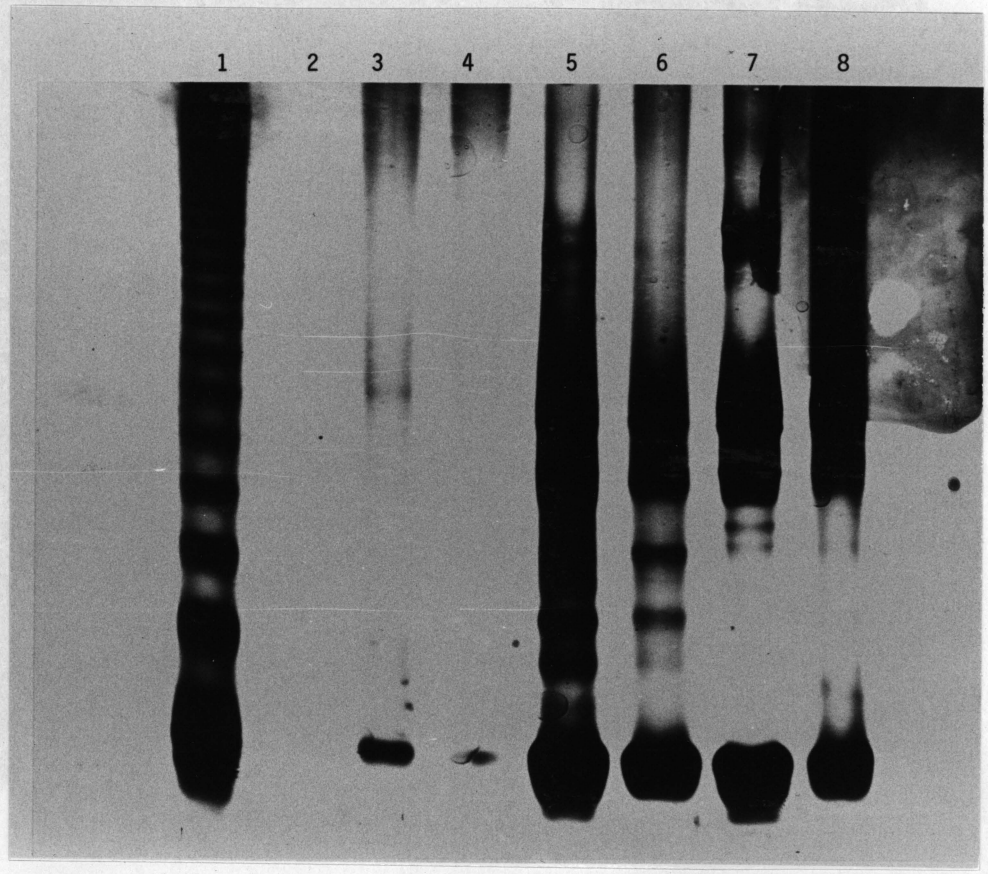

FIGURE 26. 16CM, 15% acrylamide DOC-PAGE gel, heavy stain. Lanes: 1) Standard, Salmonella LPS; 2) Empty; 3) USDA LPS II; 4) HC LPS II; 5) USDA (partially induced) LPS; 6) USDA (IND) LPS II; 7) USDA LPS I; 8) HC LPS I. See text for explanation of figure and destaining procedure.

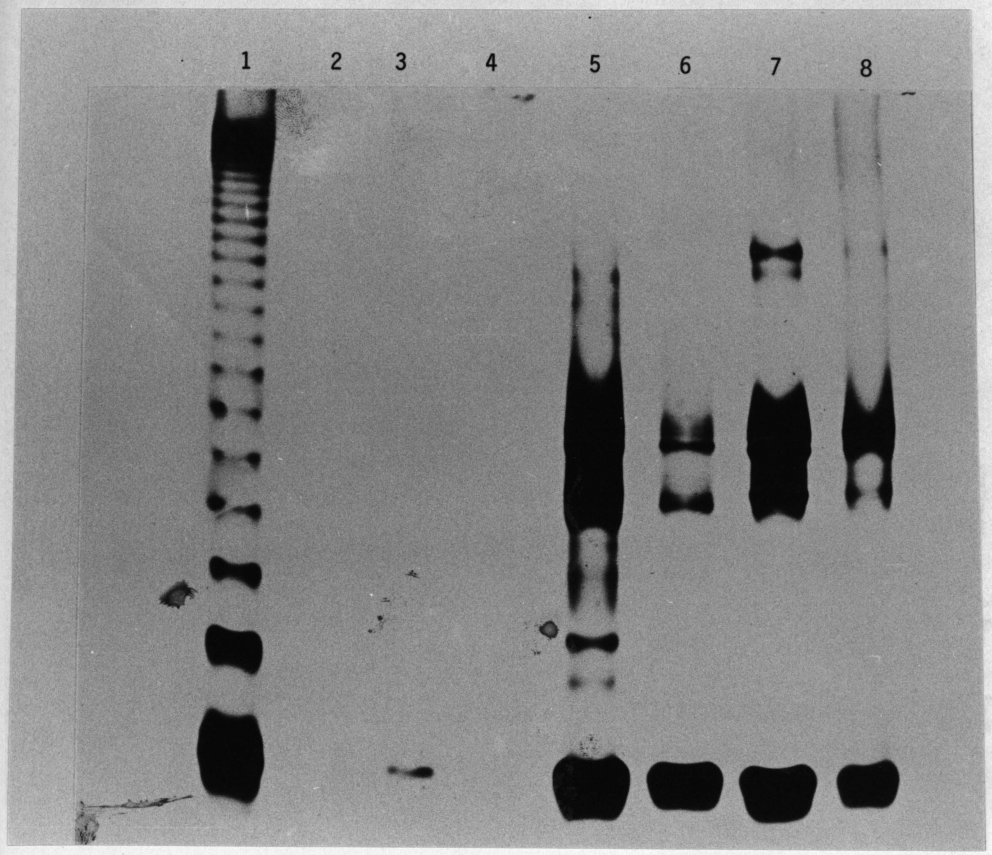

FIGURE 27. Same as figure 26, medium stain.

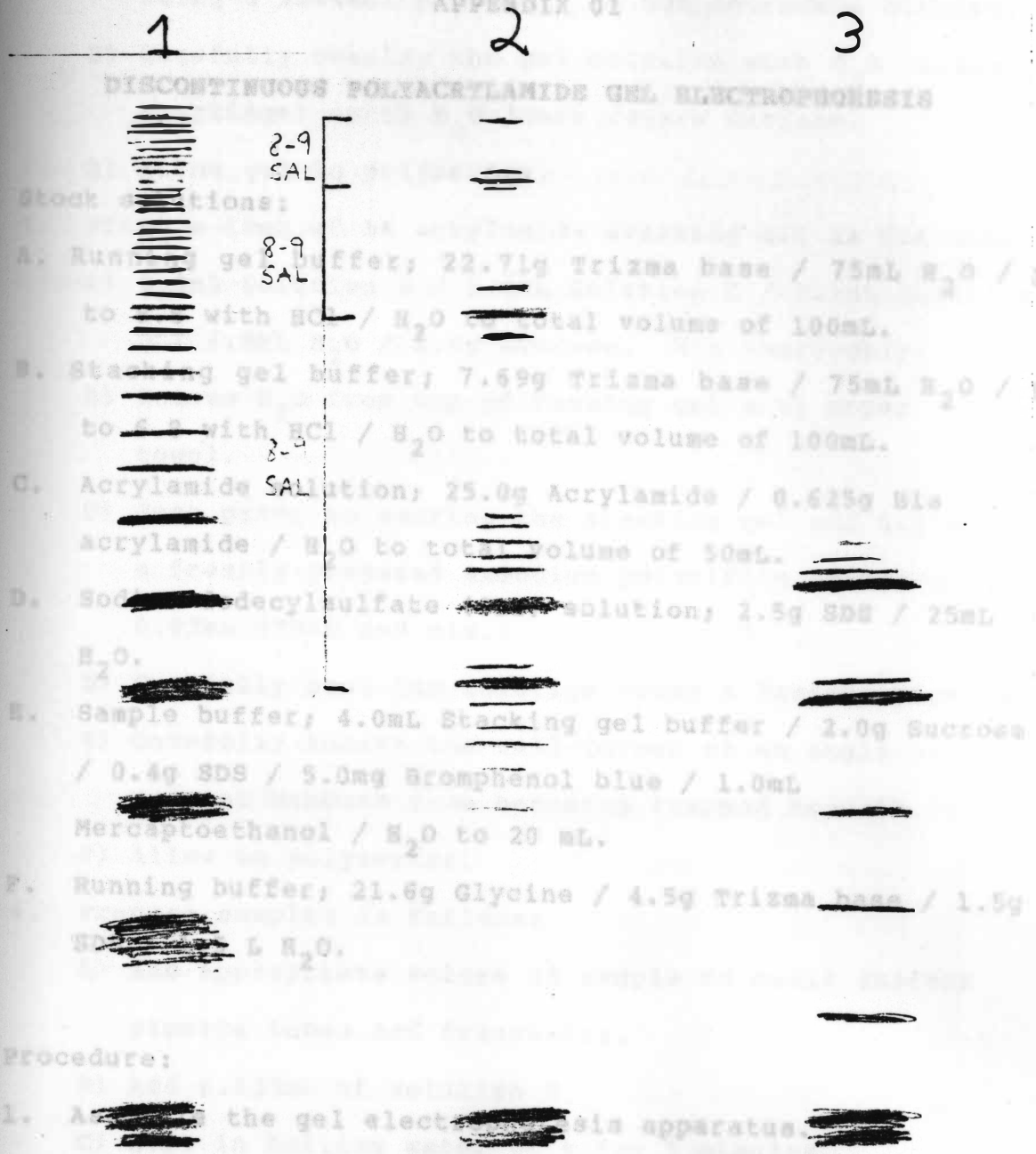

FIGURE 29. Composite drawing of three lanes from the 16cm, 15% acrylamide gel shown in figures 26, 27, and 28. The drawing was produced from the photos shown in figures 26-28 as well as others not shown. Lanes: 1) Standard, LPS from Salmonella (lane 1 in figure 26); 2) USDA LPS I (lane 7 in figure 26); 3) USDA (IND) LPS II (lane 6 in figure 26).

APPENDIX 01

DISCONTINUOUS POLYACRYLAMIDE GEL ELECTROPHORESIS

Stock solutions:

A. Running gel buffer; 22.71g Trizma base / 75mL H_2O / pH to 8.8 with HCl / H_2O to total volume of 100mL.

B. Stacking gel buffer; 7.69g Trizma base / 75mL H_2O / pH to 6.8 with HCl / H_2O to total volume of 100mL.

C. Acrylamide solution; 25.0g Acrylamide / 0.625g Bis acrylamide / H_2O to total volume of 50mL.

D. Sodium dodecylsulfate (SDS) solution; 2.5g SDS / 25mL H_2O.

E. Sample buffer; 4.0mL Stacking gel buffer / 2.0g Sucrose / 0.4g SDS / 5.0mg Bromphenol blue / 1.0mL Mercaptoethanol / H_2O to 20 mL.

F. Running buffer; 21.6g Glycine / 4.5g Trizma base / 1.5g SDS / 1.5 L H_2O.

Procedure:

1. Assemble the gel electrophoresis apparatus.
2. Prepare 25mL of a 12% acrylamide running gel as follows;
   A) 5.0mL Solution A / 6.0mL Solution C / 0.25mL Solution D / 13.75mL H_2O / 2.5g Sucrose. Mix thoroughly.
   B) Just prior to pouring the gel add 0.05mL of a freshly prepared 10% ammoniun persulfate solution and 0.02mL of TEMED to the above solution and mix.
   C) Carefully pour the solution into the gel apparatus

using a Pasteur pipette. Do not introduce bubbles.

D) Carefully overlay the gel solution with H_2O (using a syringe) until H_2O justs covers surface.

E) Allow gel to polymerize.

3. Prepare 10mL of 5% acrylamide stacking gel as follows;

A) 2.0mL Solution B / 1.0mL Solution C / 0.1mL Solution D / 6.9mL H_2O / 1.0g Sucrose. Mix thoroughly.

B) Remove H_2O from top of running gel with paper towel.

C) Just prior to pouring the stacking gel add 0.05mL of a freshly prepared ammonium persulfate solution and 0.02mL TEMED and mix.

D) Carefully pour the solution using a Pasteur pipette.

E) Carefully insert the well-former at an angle to prevent bubbles from becoming trapped beneath it.

F) Allow to polymerize.

4. Prepare samples as follows;

A) Add appropriate volume of sample to small Pasteur pipette tubes and freeze-dry.

B) Add 0.025mL of solution E

C) Heat in boiling water bath for 5 minutes.

D) Fill the upper reservoir of the gel apparatus with solution F and remove the well-former.

E) Using a syringe, apply the samples to the appropriate wells.

5. Run the gel as follows;

A) Fill the lower reservoir with solution F and

completely assemble the apparatus.

B) Run at constant current of 20ma/gel until the dye has reached the bottem of the gel.

6. Stain the gel for the appropriate macromolecules.

Note: For Triton X100 gels substitute and amount of Triton X100 equal to that of SDS.

For sodium deoxycholate (DOC) gels the SDS is omitted and 0.5% DOC is used in the stacking and running gels and 0.25% DOC is used in the running buffer. The sample buffer contains 175mM Trizma base, 10% glycerine, and 0.25% DOC, pH 6.8. The gels are run at 18ma of constant current through the stacking gel and 25ma of constant current through the running gel. The running of the sample should be preceded by pre-electrophoresis. Add a small amount of sample buffer to two wells at different ends of the gel and run at 20ma until the dye has passed through the gel. Discard the running buffer and wipe the electrodes clean. Add fresh running buffer and run as described above. The gel should be used immediately after pre-electrophoresis.

APPENDIX 02

SILVER STAINING PROCEDURE FOR LIPOPOLYSACCHARIDES

Stock solutions:

A. 0.1 M NaOH solution.

B. Fixing solution; 250mL isopropanol / 100mL glacial acetic acid / 650mL H_2O.

C. 20% $AgNO_3$ solution.

D. Sodium metaperiodate solution; 1.05g $NaIO_4$ / 4mL Fixing solution / 150mL H_2O.

E. Ammonium hydroxide solution; 115mL H_2O / 28mL solution A / 5mL solution C / 1mL fresh concentrated NH_4OH / mix and add concentrated NH_4OH dropwise (while stirring) until brown precipitate <u>just</u> disappears.

F. Citric acid solution; 0.05g citric acid / 0.5mL of 37% formaldehyde / 500mL H_2O.

Procedure:

1. Place gel in enough of solution B to cover and allow to shake overnight. Drain and rinse with H_2O. Drain again.
2. Place in solution solution D for 5 minutes while shaking.
3. Remove the gel from solution and add H_2O, allow to shake for 30 minutes. Repeat this step two more times.
4. Place gel in solution E for 10 minutes with shaking.
5. Remove the gel from solution and add H_2O, allow to

shake for 30 minutes. Repeat this step two more times.

6. Place gel in solution F until bands develop. Remove from solution and rinse three times with H_2O.

Note: For DOC gels the fixing solution is composed of 40% ethanol and 5% acetic acid. The gels are left in fixing solution for at least two days with at least two changes of solution. The oxidizing solution (solution D) is 0.7% $NaIO_4$ in 40% ethanol and 5% acetic acid.

APPENDIX 03

PROTEIN STAINING PROCEDURE

Stock solutions:

A. Staining solution; 500mL H_2O / 500mL Methanol / 100mL Glacial Acetic acid / 0.1% Coomassie Blue.

B. Destaining solution; 800mL H_2O / 100mL Methanol / 100mL Glacial Acetic acid.

Procedure:

1. Put the gel in enough solution A to cover and allow to shake for several hours to overnight.
2. Remove to enough solution B to cover and allow to shake for 1 hour. Drain and rinse with H_2O. Add more solution B and repeat until only bands show blue color.

APPENDIX 04

LOWRY PROTEIN ASSAY

Reagents:

A. Alkaline copper reagent; 1mL of 1% $CuSO_4*5H_2O$ / 1mL of 2% Sodium tartate / 98mL of 2% $NaCO_3$ in 0.1 N NaOH.

B. Phenol Reagent Solution, 2 N (Folin-Ciocalteau).

Procedure:

1. Set up a set of standard tubes using 1mg/mL BSA.
2. To 0.5mL of sample and standards add 3.0mL of reagent A. Mix. Allow to stand for 10 minutes at room temperature.
3. Add 0.2mL of reagent B. Mix. Allow to stand for 30 minutes at room temperature.
4. Measure absorbance at 650nm.

APPENDIX 05

ANTHRONE ASSAY FOR HEXOSE

Reagents:

A. Anthrone reagent; 0.2% Anthrone in concentrated H_2SO_4.

Procedure:

1. Prepare a set of standards with a 1mg/mL solution of glucose.
2. To 0.5mL of sample and standards add 1.0mL of reagent A. Vortex.
3. Heat the tubes in a boiling water bath for 5 minutes.
4. Read absorbance at 620nm.

Note: For qualitative assay where the samples contain a high concentration of sugar, step 3 may be omitted.

APPENDIX 06

2-KETO-3-DEOXYOCTONIC ACID (KDO) ASSAY

Reagents:

A. 0.4N H_2SO_4

B. 0.04N HIO_4 in 0.4N H_2SO_4

C. 2% $NaAsO_2$ in 0.5N HCl

D. 0.3% Thiobarbituric acid

Procedure:

1. Prepare a set of standards from a 0.1mg/mL solution of KDO.
2. To 0.2mL of sample and standards add 20uL of reagent A. Vortex. Heat in boiling water bath for 30 minutes.
3. Add 250uL of reagent B. Vortex. Let stand at room temperature for <u>at least</u> 40 minutes.
4. Add 500uL of reagent C. Vortex. Let stand at room temperature for 5 minutes.
5. Add 2.0mL of reagent D. Vortex. Place in hot water bath for 20 minutes.
6. Read absorbance immediately at 548nm.

APPENDIX 07

PYRUVIC ACID ASSAY

Reagents:

A. 500 umoles of 2,4-dinitrophenylhydrazine (DNP) in 100 mL of 2.0N hydrochloric acid. Make fresh each time.

B. 2.2N NaOH.

C. 2.0N HCl.

D. Toluene.

E. 10.0% $NaCO_3$.

Procedure:

1. Prepare a set of standards from a 0.5mg/mL solution of pyruvic acid.
2. To 0.2mL of sample and standards add 0.3mL of reagent C.
3. Heat in sealed tubes for 3 hours at 100°C.
4. Remove and add 0.1mL of reagent A. Vortex. Let stand at room temperature for 30 minutes.
5. Add 0.6mL of reagent D and vortex.
6. Remove the bottom layer and discard. To the top layer add 0.6mL of reagent E and vortex.
7. Discard the top layer and to the bottem layer add 0.4mL of H_2O and 1.0mL of reagent B. Vortex.
8. Read absorbance at 416nm.

APPENDIX 08

ACETYL GROUP ASSAY

Reagents:

A. 2.0M hydroxylamine-hydrochloride (store at $4^{o}C$).

B. 3.5M NaOH.

C. 1 part concentrated HCl in 2 parts H_2O.

D. 0.37M $FeCl_3$ in 0.1N HCl.

E. 0.5mg/mL glucose pentacetate in methanol.

Procedure:

1. Prepare a set of standards from reagent E.
2. Mix equal parts of reagent A and reagent B just before use.
3. To 0.4mL of sample and standards add 0.4mL of the reagent made in step 2. Vortex and let stand at room temperature for at least 1 minute.
4. Add 0.2mL of reagent C and vortex.
5. Add 0.2mL of reagent D and vortex.
6. Read absorbance at 540nm.

APPENDIX 09

URONIC ACID ASSAY

Reagents:

A. 0.0125M $Na_2B_4O_7*10H_2O$ in concentrated sulfuric acid.

B. 0.15% m-hydroxydiphenyl in 0.5% NaOH.

Procedure:

1. Prepare a set of standards from a 0.1mg/mL solution of glucuronic acid.
2. To 0.2mL of sample and standards add 1.2mL of reagent A.
3. Vortex and heat in boiling water bath for 5 minutes.
4. Remove and cool in ice bath for 2 minutes.
5. Add 0.02mL of reagent B and vortex. Allow to stand at room temperature for 5 minutes.
6. Read absorbance at 520nm.

Note: If samples turn brown in step 5 prepare a duplicate set of samples with 0.5% NaOH but without m-hydroxydiphenyl. Run as for others and read absorbance at 520nm. When finished subtract the duplicate set readings from the regular set readings.

APPENDIX 10

ACETYLATION PROCEDURE

Solutions and reagents:

A. 1.0mg/mL inositol.

B. Standard sugar solution containing 1.0mg/mL of each sugar.

C. 2.0M trifluoroacetic acid (TFA)

D. 10mg/mL sodium borohydride (or Na borodeuteride) in 1.0M NH_4OH

E. Glacial acetic acid.

F. MeOH/HAc (9/1).

G. Methanol.

H. Pyridine.

I. Acetic anhydride.

J. Chloroform.

K. Dichloromethane.

Procedure:

1. Freeze dry the standard sugars and samples.
2. Prepare a 1.0mg/mL solution of the sample polysaccharide.
3. Determine the percent hexose of sample using the anthrone assay (see appendix 05).
4. Prepare the standard sugar solution. Store in freezer after each use.
5. Place a volume of the sample equivalent to not more than

250ug of hexose in a screw-cap test tube. In another tube place 100uL of the standard sugar solution. Add 20uL of inositol solution to each tube. Freeze-dry sample and standards.

6. Add 500uL of solution C to each tube. Seal with teflon-lined screw cap and heat at 121^{o}C for 2 hours.
7. Remove tubes and blow-dry at 40-50^{o}C.
8. Add 250uL of solution D, mix and allow to stand for 1 hour at room temperature.
9. Add 50uL of glacial acetic acid and observe. Vigorous bubbling should take place. Repeat two more times.
10. Add 500uL of solution F to each tube then blow-dry using filtered air. Repeat 4 times.
11. Add 500uL of methanol to each tube then blow-dry using filtered air. Repeat 4 times.
12. Add 50uL of pyridine and 50uL of acetic anhydride to each tube. Mix, seal the tubes with teflon-lined cap and heat at 121^{o}C for 30 minutes.
13. Cool tubes on ice and blow-dry at room temperature. Extract by adding 500uL of H_2O and 500uL of chloroform. Vortex. Centrifuge in Adams analytical centrifuge for 5 minutes. Remove the chloroform layer with a Pasteur pipette and transfer to another tube. Reextract water layer with 500uL of chloroform and combine the chloroform layers from the two extractions. Blow-dry chloroform layers with filtered air.
14. Analyze by dissolving in 20 to 100uL of dichloromenthane

and injecting 1 to 5uL of this solution into the gas chromatograph.

Note: These additional steps are necessary for samples containing a high percentage of 2-keto-3-deoxyoctonate (KDO).

1. To 400-500ug of freeze-dried sample add 1.0mL of 1% acetic acid and 20uL of solution A.
2. Heat at 100^{o}C for 2 hours.
3. Blow-dry 30 minutes to 1 hour at 40^{o}C. It must be dry.
4. Add 150ul of solution D and leave at room temperature for 1 hour.
5. Add 50uL of glacial acetic acid and shake. Add 50uL more glacial acetic acid.
6. Add 500uL of solution F and blow-dry at 40^{o}C. Repeat 4 times.
7. Add 500uL of methanol and blow-dry at 40^{o}C. Repeat 4 times.
8. Begin regular acetylation procedure at step 6.

APPENDIX 11

ELISA

Buffers:

A. Coating buffer; 1.59g Na_2CO_3 / 2.93g $NaHCO_3$ / 0.2g NaN_3 / 1L H_2O (final pH, 9.58).

B. Phosphate-buffered saline with tween (PBS-tween); 8.0g NaCl / 0.2g KH_2PO_4 / 2.2g $Na_2HPO_4*7H_2O$ / 0.2g KCl / 0.2g NaN_3 / 0.5mL Tween-20 / 1L H_2O (final pH, 6.60).

C. Substrate buffer; 7.51g Glycine / 0.2g $MgCl_2*6H_2O$ / 0.136g $ZnCl_2$ / 1L H_2O (final pH, 10.5 with NaOH).

Procedure:

1. Remove cells from working slant with 3mL of 0.85% saline solution and heat treat for 15 minutes in boiling water bath.
2. Dilute to O.D. of 0.5 at 600nm with buffer A. Add 0.1mL of this suspension to each well of microtiter plate.
3. Incubate overnight at $4^{o}C$.
4. Empty excess and wash 3 times with buffer B, leaving the buffer in the wells for 5 minutes the last two times.
5. Add 0.1mL of antisera diluted in buffer B to the appropriate wells.
6. Incubate for 1 hours at room temperature.
7. Repeat step 4 (washing).
8. Add 0.1mL of goat anti-rabbit*alkaline phosphatase

diluted in Buffer B (1/1000) to each well.

9. Incubate for 3 hours at 37°C.
10. Repeat step 4.
11. Add 0.1mL of p-nitrophenyl phosphate substrate diluted in buffer C (1mg/mL) to each well used.
12. Allow to develop a measurable amount of yellow color at room temperature.
13. Stop the reaction with 50uL of 3M NaOH.
14. Remove contents of each well to test tubes containing 2mL of H_2O and read absorbance at 405nm.

Note: Negative control wells may be established by leaving out the antibodies.

APPENDIX 12

ANTIBODY DEVOLOPMENT

Buffer: Phosphate buffered saline (PBS); 10mM $NaPO_4*7H_2O$ / 150mM NaCl.

Procedure:

1. The antigens were grown in YEM to an O.D. of ca. 1.85 and harvested by centrifugation (10,000rpm for 10 minutes).
2. Pellets were resuspended in PBS and stored at $4^{o}C$ to be used as stock inoculum.
3. Stock was diluted before each injection with PBS.
4. Rabbits were intravenously injected in the marginal ear vein according to the following schedule; Day 1--0.5mL of stock inoculum diluted to 1.0 abs at 620nm
   Day 2--1.0mL of stock at 1.2 abs at 620nm
   Day 3--1.0mL of stock at 1.6 abs at 620
   Day 8--1.0mL of stock at 1.6 abs at 620nm
   Day 9--1.0mL of stock at 2.1 abs at 620nm
   Day 10-1.0mL of stock at 2.1 abs at 620nm
   Day 18-Rabbits killed and blood removed.

APPENDIX 13

MODIFIED LIPOPLYSACCHARIDE EXTRACTION PROCEDURE

Solutions and reagents:

A. EDTA solution; 0.05M $Na_2HPO_4*7H_2O$ / 0.005M EDTA / 0.05% NaN_3 (final pH, 7.0).

B. Phenol.

C. 0.04M $MgCl_2*6H_2O$.

d. Enzymes; lysozyme, RNase, DNase.

Procedure:

1. Place 150mL of solution A in a 600mL beaker and place in cold room on crushed ice.
2. Add 20-30g of bacterial pellet and stir to suspend.
3. Blend the mixture in a Waring blender in the cold room for one minute. Let stand one minute. Repeat two more times.
4. Sonicate the mixture in the cold room using a Bronson sonic disruptor with the largest probe. Sonicate for 30s at 70% maximum power. Pause for 1 minute. Repeat this sonication procedure 10 times in total.
5. Add 15mg of lysozyme and allow to stir overnight in cold room ($4^{o}C$).
6. Heat the mixture to $37^{o}C$ with stirring and allow to stir for 20 minutes.
7. Repeat step 4.
8. Add 50mL of solution C, 10mg of DNase and 100mg of

RNase.

9. Incubate for 10 minutes at 37°C followed by a 10 minute incubation at 60°C.
10. Heat mixture to 65°C and add to 200mL of 90% phenol which has been preheated to 65°C.
11. Allow mixture to stir at 65°C for 15 minutes. Cool on ice for 15 minutes. Centrifuge at 9150xg for 20 minutes.
12. Remove the water layer to a separate flask. Add 200mL of H_2O, which has been preheated to 65°C, to the phenol layer. Repeat step 11. Combine water layers and reduce volume by rotary evaporation.
13. Dialyze against H_2O. Reduce volume as before and then freeze dry.

APPENDIX 14

YEAST EXTRACT MEDIUM

Ingredient	Quantity
K_2HPO_4	0.5 g/L
$MgSO_4*7H_2O$	0.2 g/L
NaCl	0.1 g/L
Mannitol	5.0 g/L
Gluconic acid	5.0 g/L
Yeast extract	0.5 g/L

Adjust the pH to 6.7-7.0 with HCl before autoclaving.

Note: For solid medium add 15.0 g/L of agar before autoclaving.

For induction medium add 3 mg/L of apigenin before autoclaving.

APPENDIX 15

B-GALACTOSIDASE ASSAY

Solutions and reagents:

A. Sample buffer; 16.1g $Na_2HPO_4*7H_2O$ / 5.5g $NaH_2PO_4* H_2O$ / 0.75g KCl / 0.246g $MgSO_4*7H_2O$ / 2.7mL B-mercapto-ethanol / 1.0L H_2O (final pH,7.0).

B. Chloroform.

C. Substrate solution; 40mg o-nitrophenyl-B-D-galactopyranoside in 10mL 0.1M phosphate buffer (ph, 7.0).

D. 1.0M sodium carbonate.

Procedure:

1. To 0.5mL of culture sample add 0.5mL of solution A and 0.1mL of chloroform. Vortex for 10 seconds.
2. Add 0.2mL of solution C and vortex briefly.
3. Incubate at $28^{o}C$ for a measured time. The time of incubation should be long enough for the development of sufficient yellow color for measurement with a spectrophotometer. Too much color development should be avoided. This "incubation time" will be represented by the symbol T in the assay equation.
4. Add 0.5mL of 1.0M sodium carbonate to stop the reaction. Vortex briefly.
5. Centrifuge for 15 minutes in an Adams analytical centrifuge.
6. Remove supernatent with a Pasteur pipette and measure

the absorbance at 420nm.

7. Calculate the activity with the following equation;

$$\text{ACTIVITY UNITS} = \frac{\text{O.D. @ 420nm}}{T \times V \times \text{O.D. @ 600nm}}$$

where T=time of incubation (see step 3) and V=volume of sample used.

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