Report No. 4

Preparation and Research on Meningococcal Vaccines

Final Report

M. R. Hilleman, Ph.D.

March 1, 1972

Supported by
U.S. Army Medical Research and Development Command
Washington, D.C. 20314

Contract No. DADA17-71-C-1029

Merck & Co., Inc
Rahway, N. J. 07065

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13. ABSTRACT

A contract was arranged between the U.S. Department of Defense and Merck & Co., Inc. to prepare on a feasability basis 20 gms. of meningococcal polysaccharide vaccine, type C and on a best-effort basis up to 20 gms. of meningococcal polysaccharide vaccine, type A. The methodology for general vaccine preparation and control testing was established by the U.S. Army and was supplied to Merck & Co., Inc. in the form of specifications. The duration of the contract was from October 6, 1970 to December 31, 1971. The contractual committment was met on schedule.

Slightly more than 20 gms. of meningococcal polysaccharide vaccine, type C was produced (413,350 x 50 mcg doses). The material was produced as eight separate lots, each of which met the specifications of the contract. They were delivered to Dr. M. Artenstein, Walter Reed Army Institute of Research, as lyophilized formulations. Release protocols describing the preparation and control testing procedures and results for each of the lots accompanied the vaccines. A summary of the lot numbers and quantities delivered is as follows:

DD FORM 1 NOV 68 1473 REPLACES DD FORM 1473, 1 JAN 64, WHICH IS OBSOLETE FOR ARMY USE.

Security Classification

13. Abstract - (cont'd.)

Material	Lot Number	No. Doses/Lot	Total No. Doses
Meningococcal Polysaccharide Vaccine, Type C	419/C-A258	48,600	
	420/C-A259	49,700	
	421/C-A260	58,000	
	422/C-A261	55,550	
	423/C-A262	48,950	
	424/C-A263	34,650	
	426/C-A265	64,950	
	438/C-A406	52,950	413,350

Additionally, slightly more than 5 gms. of meningococcal polysaccharide vaccine type A was produced (101,500 x 50 mcg doses). The material was produced as two separate lots, each of which met the specifications of the contract. They were delivered to Dr. M. Artenstein, WRAIR, as lyophilized formulations. Release protocols describing the preparation and control testing procedures and results for each of the lots accompanied the vaccines. A summary of the lot numbers and quantities delivered is as follows:

Material	Lot Number	No. Doses/Lot	Total No. Doses
Meningococcal Polysaccharide Vaccine, Type A	439/C-A453	48,000	
	440/C-A455	53,500	101,500

In addition to the vaccines, sufficient diluent (pyrogen-free distilled water without preservative) for resuspension of the lyophilized vaccines was provided in the form of four separate lots (548,500 x 50 mcg doses). These were sent to Dr. M. Artenstein, WRAIR. Certificates of Analysis, presenting the results of control testing for each of the lots were included with the diluent shipments. A summary of the lot numbers and quantities delivered is as follows:

Material	Lot No.	No. Rehydration Doses
Diluent (Pyrogen-free Distilled Water)	440064/20438/C-A467	
	437717/18327/C-A484	
	437718/18328/C-A485	
	440065/20440/C-A590	548,500

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Abstract

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	421/C-A260	58,000	
	422/C-A261	55,550	
	423/C-A262	48,950	
	424/C-A263	34,650	
	426/C-A265	64,950	
	436/C-A406	52,950	413,350

Additionally, slightly more than 5 gms. of meningococcal polysaccharide vaccine type A, was produced (101,500 x 50 mcg doses). The material was produced as two separate lots, each of which met the specifications of the contract. They were delivered to Dr. M. Artenstein, WRAIR, as lyophilized formulations. Release protocols describing the preparation and control testing procedures and results for each of the lots accompanied the vaccines. A summary of the lot numbers and quantities delivered is as follows:

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Material	Lot No.	No. Rehydration Doses
Diluent (Pyrogen-free Distilled Water)	440064/20438/C-A487 437717/18327/C-A484 437718/18328/C-A485 440065/20440/C-A590	548,500

Preparation and Research on Meningococcal Vaccines

A contract between Headquarters, U.S. Army Medical Research and Development Command and Merck & Co., Inc. to prepare 20 gm. of meningococcal polysaccharide vaccine, type C and up to 20 gm. of meningococcal polysaccharide vaccine, type A, on a best-effort basis, was approved on October 6, 1970 for a time period of 15 months (December 31, 1971). This report constitutes the final report for the project.

A. Meningococcal Polysaccharide Vaccine, Type C

1. Growth and Isolation Studies

a. Growth studies. Production of Type C meningococcal polysaccharide vaccine started the week of 12/21/70 in shake-flasks while awaiting the construction of a pathogen facility to house the 100-liter fermentor which ultimately was to be used in the fermentation of the organisms. The culture used was Neisseria meningitidis C11 obtained from WRAIR. Two-liter flasks containing 1 liter of Watson-Scherp medium on a rotary shaker served as the production vessels. Ninety-liter batches were prepared in this manner each week, terminating the week of 5/31/71.

The 100-liter fermentor (Chempac, obtained from Squibb) became operative on 5/10/71. Through the week of 6/24/71, a total of 15 batches of 100-liters each was produced. The first batch was discarded because the cell yield was low because of insufficient agitation during growth and two other batches were lost to contamination and mechanical failure. Thus, a total of 12 batches was delivered for chemical isolation. The fermentor procedure varied from the shake-flask procedure in that a pasteurization step of 60°C for 30 minutes was used at the end of the fermentation cycle and prior to the Cetavlon precipitation step. Also, antifoam was used in the fermentor.

Table I lists the shake-flask production batches and Table II lists the fermentor batches.

(1) Shake-Flask Procedures

(a) Medium and Inoculum

A tube containing the lyophilized culture of Neisseria meningitidis, type C-11 was opened and Watson-Scherp medium was added. The culture was streaked on Mueller-Hinton agar plates and incubated at 37°C in a candle jar for 18 hours. The organisms from the agar plates were transferred to fresh plates of the same medium and incubated at 37°C in a candle jar for 18 hours. Ten ml of medium were added to each plate; the cultures were suspended in the broth and aliquoted in 2.0 ml amounts distributed into 5.0 ml screw-cap vials and frozen at -70°C as master pre-seed stock. The frozen culture was used to inoculate Mueller-Hinton agar plates which were

incubated for 16 hours at 37°C in a candle jar. Growth from a plate was suspended in 5 ml of Watson-Scherp medium and transferred to a 250 ml Erlenmeyer flask containing 60 ml of Watson-Scherp medium. Each 250 ml flask inoculated was incubated for 4 hours at 37°C on a rotary shaker at 120 rpm. At the end of 4 hours, 10 ml of this culture were transferred into another 250 ml flask containing 60 ml of Watson-Scherp medium and incubated for 4 hours at 37°C at 120 rpm. The contents of each 250 ml flask were used to inoculate 1-liter of Watson-Scherp medium in a 2-liter Erlenmeyer flask.

(b) Incubation

The 2-liter shake-flasks were incubated for 16 hours at 37°C on a rotary shaker at 120 rpm. At the end of 16 hours incubation, one-half of the flasks were picked at random and checked microscopically and with a gram-stain. The pH was adjusted to 6.8 with 1.0N NaOH(~ 2 ml per flask) and 10 ml of 10% Cetavlon were added per 2-liter flask. The 2-liter flasks were shaken for at least 1 hour at 120 rpm to allow time for the detergent to sterilize the culture. The contents of the flasks were pooled in 5-gallon jugs and permitted to remain stationary for an additional 1/2 hour. Sterility checks were performed on the material in the 5-gallon jugs.

(c) Fermentation Results

The average yield of cell paste was about 1.5 g/liter in batches 1-13. A study of the effect of aeration indicated a shaker speed increase to 200 rpm markedly increased cell weight. Thus, from batch 14 through 42, the increased aeration produced cell paste yields of approximately 2.4 g/liter. The shaker used had a throw of 2 inches and the 200 rpm change also was applied to the inoculum flasks.

Table I tabulates the cell weights on all the shake-flask batches produced.

(2) One-Hundred Liter Fermentor Procedure

(a) Inoculum

A frozen culture of Neisseria meningitidis C-11 was used to inoculate Mueller-Hinton agar plates which were incubated for 16 hours at 37°C in a candle jar. Growth from a plate was suspended in 5 ml of Watson-Scherp medium and transferred to a 250 ml Erlenmeyer flask containing 60 ml of Watson-Scherp medium. Each flask (total of 20) was incubated for 4 hours at 37°C on a rotary shaker at 200 rpm. At the end of 4 hours, the contents of four

250 ml flasks were used as inoculum for a 2-liter flask containing 800 ml of Watson-Scherp medium. Five 2-liter flasks were incubated for 3-4 hours at 37°C at 200 rpm and the 5 liters were used as inoculum for the 100-liter fermentor.

(b) Medium

The following was added to the fermentor and sterilized for 60 minutes at 121°C:

400 ml of 8% UCON LB625 lubricant
317 mg Phenol Red
265 gm Na_2HPO_4
brought up to 88 liters with distilled water

The LB625 lubricant was pre-sterilized for 60 minutes at 121°C before addition to the fermentor.

The following concentrate was filter-sterilized into the sterile fermentor:

Casamino Acids	1080	gm
Dextrose	540	gm
$MgSO_4 \cdot 7H_2O$	70.2	gm
KCl	9.9	gm
L-Cysteine·HCl monohydrate	2.178	gm

taken up to 9 liters with distilled water

A Horm press using D-8 filter pads was used as a prefilter and a 293 mm Millipore used as the final filter.

(c) Fermentation Results

The fermentation cycle used for Neisseria meningitidis C-11 was a period of 16 hours. Aeration was found to be critical and an air flow of 2 CFM under 1 PSI with an agitator setting of 75 was used for all batches delivered. The average cell yield was 2.8 g/liter, with a 16 hour cycle using a 5% inoculum with temperatures controlled at 37°C ± 1°C.

At the end of the fermentation cycle the batch was pasteurized for 1/2 hour at 60°C. The broth was adjusted to a pH of 6.8 and then harvested into 5-gallon jugs containing 10 ml of 10% Cetavlon per liter of broth. The material was delivered for recovery of final product after being in contact with Cetavlon for a minimum time of 1 hour.

Figure 1 relates O.D., pH and glucose utilization of Neisseria meningitidis C-11 during a typical 16 hour cycle in the 100-liter fermentor.

b. Isolation Studies

(1) Process Selection

Three variations of the isolation method were available and each one was tried. The most successful method, the one published by the WRAIR group, Sanford Berman, et al., [Infection and Immunity 2, 640-3 (1971)] was adopted and used to produce all of the delivered material. Personal discussions with these authors during the course of the work proved to be very helpful.

Another isolation method studied was described in the Protocol received directly from Dr. J. P. Lowenthal. It differed from the adopted method in only one respect; before the final alcohol fractionation steps, the product was dissolved in saturated sodium acetate solution, precipitated with alcohol, redissolved in water and reprecipitated with alcohol. This may have converted the product from the calcium salt to the sodium salt but it was not analyzed. This method was tried on shake-flask batches 9-15. The average yield was 1.04 g/100 liters of broth and the products were pyrogenic. Also, the isolation method described by E. C. Gotschlich, et al., J. Exp. Med. 129, 1349-1364, 1969 was carried out. It differed from the method adopted in three respects. First, the sodium to calcium conversion step mentioned above was included and the product was analyzed and found to be the sodium salt. Second, the crude polysaccharide was dissolved in sodium acetate solution instead of water and subjected to ultracentrifugation before the chloroform emulsification steps. Third, the emulsifications were done on this sodium acetate solution instead of on a water solution. This method was tried on combined shake-flask batches 3 and 5. The yield was 1.65 g/100 liters and again the product was pyrogenic.

None of the products of these alternate processes were submitted for formulation.

(2) Process

Step #1. Wet Cell Collection

The broth was centrifuged in a turbine-driven Sharples centrifuge using a number 1-H standard clarifier bowl and revolving at 50,000 RPM and a flow rate of 30-40 liters/hour. The centrifugation of the broth was followed with a wash of 1 liter of pyrogen-free water. The clear filtrate was

discarded and the insoluble cake weighed and frozen.

Step #2. Water Wash of Cells

Batches of frozen precipitate were combined as shown in Table I, suspended in 600 ml of pyrogen-free (PF) water and warmed to 5°C. The suspension was stirred with a Sorvall Omni-Mixer at a speed setting of 2 for 20 min. at 5°C. The mixture was centrifuged in 500 ml stainless steel cups in a Lourdes Model LRA centrifuge with a 3RA head at 6,000 RPM for 20 min. at 5°C. The wash was discarded.

Step #3. Extraction with $CaCl_2$ Solution

Washed cells were stirred in 600 ml of 1M $CaCl_2 \cdot 2H_2O$ solution with an Omni-Mixer at a speed setting of 6 for 30 minutes. The mixture was centrifuged in the Lourdes at 6,000 RPM., 5°C, for 30 min. The clear supernate was decanted and saved. The cake was extracted three additional times by the same method except that the Omni-Mixer was operated at a speed setting of 2 for 15 min. and the time of centrifuging was 15 min., 15 min. and 40 min., respectively. All four extracts were combined and the residual insoluble material discarded.

Step #4. Ethanol Precipitation (25%)

To the combined $CaCl_2$ extracts were added 800 ml of denatured absolute ethanol (2BA). The solution was allowed to stand in the refrigerator overnight. The 25% ethanol solution was then centrifuged in the Lourdes as before and the precipitate discarded.

Step #5, Ethanol Precipitation (80%)

The clear solution from Step #4 was adjusted to 80% ethanol by the addition of 8.8 liters of 2BA. The solution was allowed to stand at 5°C for a minimum of 3 hours. The precipitate was collected by vacuum filtration using a 24 cm. Buchner funnel set with two sheets of Reeve Angel #230 paper. The insoluble cake was washed with about 200 ml of absolute ethanol followed by 200 ml acetone and was dried *in vacuo* in a desiccator over $CaCl_2$. The dried product was weighed.

Step #6. Chloroform Emulsification (Sevag)

The dry product from Step #5 was dissolved in 500 ml of pyrogen-free water and centrifuged in an International clinical centrifuge. The small amount of insoluble material was discarded. A 400 ml portion of chloroform was added and the mixture stirred with an Omni-Mixer at a speed setting of 2.0 for 30 minutes maintaining the 5°C temperature with an ice

bath during mixing. The mixture was centrifuged in the Lourdes at 6,000 RPM for 20 minutes using polyethylene bottles. The clear water layer was siphoned off from the emulsion and the water treated 3 additional times with 400 ml portions of chloroform. The clear chloroform layers were discarded and emulsions at the interface saved for future work-up.

Step #7. Second Ethanol Precipitation

To the water layer after Sevaging (400 ml) were added 1,600 ml of absolute ethanol. The suspension was allowed to stand overnight at 5°C and the precipitate collected by centrifugation in the Lourdes centrifuge as before. The precipitate from several of the batches was dried *in vacuo* for evaluation of yield at this step.

Step #8. Second Extraction with $CaCl_2$ Solution

The precipitate was extracted with portions of 0.02M $CaCl_2$ solution until a total of 300 ml of extract was obtained. Each extract was centrifuged in an International centrifuge and the final dark precipitate was discarded.

Step #9. Ultracentrifugation

The aqueous extract was clarified by centrifugation in a Beckman Model L centrifuge using a #30 head at 30,000 RPM for 2 hours at 5°C. The pellet was discarded. The clear solution, approximately 250 ml., was diluted with about 100 ml of absolute ethanol (31-35%) which produced opalescence. The solution was again clarified by ultracentrifugation and the second pellet discarded. The clear solution was diluted with sufficient ethanol to again produce opalescence (35-38%). The opalescent solution was clarified a third time by ultracentrifugation as above for 5 hrs. The third pellet was discarded. The final clear solution, approximately 363 ml., was diluted with 10 ml of neutral saturated sodium acetate solution then with absolute ethanol to a total of 3 volumes. The solution was allowed to stand and the precipitate settle out at 5°C.

Step #10. Final Product

The insoluble product was recovered by decanting as much clear solvent as possible. The suspension was transferred to a 40 ml centrifuge tube and centrifuged in an International centrifuge and the clear solution decanted. The precipitate was washed thoroughly with 2 x 40 ml of absolute ethanol followed by 2 x 40 ml of acetone. The product was dried *in vacuo* in a desiccator over $CaCl_2$. The final dried product was weighed (Table I). Samples of each of the final products

Step #10. Final Product (cont.)

were submitted for analyses. A total of 42 batches of shake-flask broth was produced and the data collected are given in Table I. The data for the 13 fermentor batches produced are given in Table II. A total of 27.4 g of product was prepared for formulation into final vaccine.

(3) Delivery of Lots for Formulation into Final Vaccine.

The final isolation product was combined into batches containing approximately 3 g and delivered for formulation into final vaccine as outlined in Table III.

(4) Analyses

Assay	Method	Specification
Sialic Acid	Svennerholm*	min. 75%
Protein	Lowry	max. 1%
Nucleic Acid	UV Spectroscopy	max. 1%
Pyrogens	3 rabbits 2.5 µg I.V. each	no more than 0.5°C average rise, no more than 1.0°C rise in any single rabbit
Molecular Weight	(a) Sephadex G-200 Chromatography	Bulk of sialic acid in void volume
	(b) Sepharose 4B** Chromatography	None

*All samples except the first two shake-flask batches were assayed against a sialic acid standard from Cal-Biochem. The specifications are N = 4.44% (98% of theory) and homogenous by chromatography.

Analytical results of all deliveries for formulation are listed in Table IV.

** Sepharose 4B Molecular Weight Determinations

One of the specifications for acceptance of polysaccharide for vaccine preparation required the polysaccharide to be in the void volume of a Sephadex G-200 column indicating a molecular weight of greater than 200,000. All of the preparations combined for shipment and further processing to the vaccine stage passed this requirement as measured by the sialic acid content of the column charge compared to the total sialic acid in the void volume. To determine the

actual molecular weight of these fractions, Sepharose 4B was used. According to literature and descriptive information from Pharmacia, the manufacturer of Sepharose, this gel would permit molecular weight determination in the expected range.

The procedure followed for the preparation of the gel and column packing was taken from booklets published by Pharmacia titled, "Sepharose Agarose Gels in Bead Form" and "Calibration Kit Instruction Manual."

Columns were prepared using the following:

Buffer - .05M Tris HCl, pH 7.4
Column Size - 2.5/45 purchased from Pharmacia
Gel - Sepharose 4B purchased from Pharmacia
Flow - Gravity 15 ml/hr with Mariotte flask adjustment
Hydrostatic Head - 140 cm
Recording Refractometer - Waters R4, cell size .07 ml., attenuation 2X
Calibration - Blue Dextran (M.W. $2x10^6$), Dextran T500 ($5x10^5$) Dextran T150 ($1.5x10^5$) sodium acetate, calcium chloride
Charge - 10 mg of sample in 3.0 ml buffer
Fraction Size - 2.4 ml
Line Holdup - 4.8 ml

The calculation of the molecular weights of the sample was determined from the formula:

$$K_{av} = \frac{Ve - Vo}{Vt - Vo}$$

Ve = elution volume
Vo = elution volume for Blue Dextran
Vt = total bed volume

Molecular weight was determined from a graph of the K_{av} value of the standards plotted against the log M_w.

The data listed in Table V were obtained for each of the batches delivered for formulation, a sample of polysaccharide produced by Squibb and a sample isolated from Fermentor Batch #3. This last sample is an example of lower molecular weight material. The broth from Batch #3 was pasteurized for 60 minutes while subsequent batches were pasteurized for 30 minutes and this may account for the lower molecular weight. This sample was not submitted for vaccine preparation.

The attached figures were made from the refractometer recording of the elution pattern of the sample.

Figure #2 - Records the Blue Dextran and T500 standards used for molecular weight calibration.
Figure #3 - Sample 00G10 refractive index recording and sialic acid assays of the individual 2.4 ml fractions.
Figure #4 - Sample 00G15 - fermentor product
Figure #5 - Sample 00G16 - shake flask product
Figure #6 - Fermentor #3 product

(5) Process Studies

Efforts were made to study the adopted process and scale-up to process 200 liters of broth.

Step #1. Wet Cell Collection

The harvesting of the broth by centrifugation was initially made after the broth was sampled and checked for the effect of treatment with Cetavlon. By this procedure, the broth stood overnight at 5°C awaiting the report of viability by the fermentation group. Sufficient evidence was obtained to indicate that sterilization was complete after 2 hours of treatment with Cetavlon. The broths were then delivered after this short standing period rather than after overnight or weekend refrigeration.

The clear centrifugate was checked for completeness of precipitation by further addition of Cetavlon. No further precipitation was observed.

Step #2.

The water wash of cells from batch 18/19 was lyophilized and analyzed. The sialic acid content was 2% (S713-15A) with a yield of 3.6g., thus, the loss at this step was negligible.

Step #3.

The process of extraction with $CaCl_2$ was evaluated by taking 5 ml of each of the extractions and treating each according to the process. The weights of each of the final 80% precipitates are recorded below:

#1 Extract (S711-5B1) - 40.5 mg - 65% of total

#2 Extract (S711-5B2) - 14.0 mg - 23% of total
#3 Extract (S711-5B3) - 6.0 mg - 10% of total
#4 Extract (S711-5B4) - 1.4 mg - 2% of total

Four extractions were continued for the processing of all batches.

Step #5

Additional ethanol was added to the filtrate after the 80% fraction was removed by filtration and dried. No further precipitation was observed.

Several batches of the crude product from this step were analyzed in order to evaluate the extent of purification obtained in the subsequent steps. The results are presented in Table VI.

Step #6

Sevaging of the crude precipitate was studied by lyophilizing an aliquot at each stage and submitting the dry sample for analysis. The results are presented in Table VII. From these data it appears that Sevaging as a protein removing step is not very efficient.

One experiment was run to study the possibility of elimination of Sevaging. A 200 mg sample of crude broth from Step #5 was dissolved in 10 ml of 2% $CaCl_2$ and processed directly through the ultracentrifuge steps. The final yield was 71 mg, protein content 0.8%, but the sample gave a pyrogen response of 1.9, 2.1 and 1.7°C. This result indicated that the Sevag procedure did remove pyrogenic impurities.

A 100 mg sample of the same fraction was dissolved in water and made to 1% Cetavlon concentration by the addition of 10% Cetavlon. The precipitate which formed was centrifuged, recovered and lyophilized. A total of 13 mg of insoluble fraction was recovered with 72 mg in the soluble fraction after dialysis to eliminate the Cetavlon. A sample of final product was treated with Cetavlon and failed to give a precipitate. This indicated that the polysaccharide did not form an insoluble Cetavlon salt. Thus, precipitation of the product from whole broth with Cetavlon is not fully understood.

Step #9

Several of the pellet fractions recovered from the opalescence treatment with water and ethanol were reworked. A 2.1g pellet from fermentor batches 8 & 10 was dissolved in 150 ml of water containing 2% 1M $CaCl_2$. To the solution were added 100 ml of

Step #9 (cont.)

absolute ethanol. This was centrifuged in the Beckman Model L centrifuge for 2 hours and to the clear soluble fraction were added 5 ml of neutral sodium acetate solution, followed by absolute ethanol to a 75% concentration. The solution was allowed to stand and the clear filtrate decanted. The insoluble fraction was washed with ethanol and acetone and dried in vacuum. The yield was 785 mg and the sample passed all of the specifications. This indicated that yields can be improved by recovering material from the ultracentrifugation pellets.

(6) Stability

Since meningococcal type A polysaccharide has been shown to break down to lower molecular weight products, reference samples of this product were analyzed by the Sepharose 4B method given in pgs.10 & 11. The observed molecular weight was unchanged after storage in the desiccator at room temperature for as long as 3 months. The samples tested were deliveries 1, 4 and 8.

2. Formulation and Testing of Final Container Vaccine.

a. Vaccine formulation. The preparation of approximately 20 grams (413, 350 x 50 mcg doses, 0.5 ml each) of lyophilized Neisseria meningitidis type C polysaccharide vaccine has been completed. The vaccine has been delivered to Dr. M. Artenstein, WRAIR, along with release protocols which describe the preparation and control testing of each of the vaccine lots. The 20 grams were distributed in eight separate lots (50 dose containers) and each passed all contract specifications as well as control safety testing.

The control testing procedures followed for release of lot 438/C-A406 are presented in Indicies 1-10. Similar procedures were utilized in the preparation and testing of the remaining seven lots (419/C-A258, 420/C-A259, 421/C-A260, 422/C-A261, 423/C-A262, 424/C-A263 and 426/C-A265).

(1) Rehydration and filtration of final isolation pool.

All glassware, metal and rubber tubing used were prepared so as to eliminate any contribution of detectable pyrogens (refer to Index 4). All work subsequent to the weighing step of the desiccated polysaccharide was performed under a recirculating, laminar flow hood (Air Control, Microvoid model IV BC). All sample and sterile rubber hose connections were flamed properly with a bunsen burner. The total number of grams of Neisseria meningitidis, type C (strain C11) polysaccharide powder was weighed in a glass beaker on a Mettler semi-microanalytical balance (model B6). Subsequently, the powder was added to

saline solution (refer to Index 5); the same solution was used to rinse the beaker and was added to the pool. The polysaccharide solution was placed on a rotary shaker (Thomas Rotating Apparatus No. 3623, setting at 3.5) for 30 minutes at 20-25°C. A Millipore 142 mm filter holder containing a sterile 0.22 micron porosity Millipore filter (GSWP) and a Millipore prefilter (AP2012450) were primed with the saline diluent (500 to 600 ml) described above (fluid and container removed). Subsequently, the polysaccharide solution was passed through the filter under filtered air pressure, into a new container. After the filtration step, the filter pad was examined for possible breaks. The bulk, filtered solution was sterility tested (Refer to Index 6).

(2) Filling of final pool.

The sterile filtered polysaccharide solution was delivered to the MSD Biological Production Laboratories for filling into final containers (Index 10). The dispensing (2.0 ml/vial) was conducted under a laminar flow hood, using aseptic techniques, into glass vials (#50276); rubber stoppers (#52531) suitable for lyophilization were inserted half way. A member of the Virus & Cell Biology staff always was present for the entire operation. An appropriate number of vials from the beginning, middle and end of the filling operation were coded and removed after the lyophilization step. The stoppered vials were placed in an electric freezer, -60° to -70°C overnight.

(3) Lyophilization of final pool.

A separate thermocouple was attached to each of four vials which were positioned in appropriate areas of the Hull lyophilization cabinet (Model 651-3PF-25F) during the operation The shelves of the unit were cooled to -40°C, after which the trays of vials were loaded into the unit. Evacuation of the chamber was performed until the pressure was down to 175 microns, at which time the lyophilization cycle was begun; the pressure eventually was down to about 48 microns. The shelf temperature was held at -40°C for 16 hours; the temperature was increased to 0°C over a 3-hr period. The shelf temperature was held at 0°C until the material temperature increased to -5°C; the shelf temperature was increased to +10°C and held. The material temperature was at +10°C on all thermocouples for a minimum of 6 hours before unloading. The total lyophilization cycle was 45-48 hours. When the run was completed, the vacuum valves were closed and argon was admitted to a pressure reading of 3". The vial stoppers were depressed internally and sufficient argon was admitted to equilibrate atmospheric pressure.

The rubber-stoppered vials were capped with aluminum seals (flip-off red plastic center) and stored at -20°C in sealed cartons until they were labelled.

(4) Assays on final container vaccine.

The assays performed on final container type C polysaccharide vaccines have been described in Indicies 1-10. Results of final container assays have been listed in Tables VIII and IX.

TABLE I. SHAKE-FLASK PRODUCTION

Week No.	Batch No.	Broth Vol.	Wet Cell Wt.	Yield		Pyrogen Test	Sialic Acid	Protein	Nucleic Acid	Sephadex G-200	Sample No.
				Step 5	Final						
1	1	28 l.	37 g.	--	0.12g.	0.2, 0.3, 0.1	80.0%	0.07%	0.3%	100 %	S711-5M
2	2	28	41	--	0.08	0.0, 0.0, 0.0	84.0	0.1	0.3	93.6%	S711-20B
3	3&5	90	151	4.2 g.	1.30	2.0, 1.2, 0.6	70.0	0.5	1.0		D712-25A
4	4&6	90	137	4.7	0.39	0.1, 0.2, 0.0	82.0	0.1	0.5	91.8%	S712-3D
5	7&8	90	125	4.4							
6	9&10	90	138	4.0	0.49	0.3, 0.0, 0.2	80.5	0.6	0.3	89.9%	S713-23C
7	11&12	95	125	5.2							
8	13	100	161	4.6	1.04	2.0, 2.1, 1.0	69.9	0.5	0.1		D713-16A
9	14&15	95	207	8.1	1.27	0.2, 0.1	74.8	0.3	0.2	94.8%	S713-26C
10	16&17	100	232	8.8	1.26	0.2, 0.2, 0.2	81.0	0.9	0.7	91.4%	S713-24B
11	18&19	100	232	9.2	1.57	0.0, 0.0, 0.0	78.7	0.2	0.3	89.5%	S713-20A
12	20&21	100	231	8.1	0.90	0.3, 0.4, 0.0	78.0	0.5	0.4	95.1%	S713-26A
13	22&23	90	222	8.2	1.40	0.2, 0.0, 0.3	77.6	0.5	0.8	92.6%	S714-1D
14	24&25	100	238	9.2	1.35	0.4, 0.0, 0.0	79.3	0.6	0.8	96.7%	S714-7C
15	26	100	220	8.9	0.82	0.1, 0.0, 0.1	75.0	0.6	0.5	95.3%	S714-15C
16	27&28	100	250	9.0	1.70	0.2, 0.3, 0.4	75.3	0.2	0.3	94.2%	S714-22C
17	29&30	100	240	7.9	2.10	1.0, 1.2, 1.4	77.2	0.3	0.5		S714-29C
	Rework				0.98	0.0, 0.1, 0.1	77.4	0.3	0.5	94.1%	S715-7B
18	31&32	100	245	9.2	1.60	0.2, 0.2, 0.3	77.1	0.5	0.5	94.6%	S715-6C
19	33&34	100	236	7.4	0.81	0.1, 0.2, 0.0	79.7	0.6	0.8	91.2%	S715-12C
20	35&36	100	230	9.0	0.79	1.1, 0.9, 0.8	75.9	0.7	0.8		D715-20C
	Rework				0.50	0.1, 0.2, 0.0	80.0	0.5	0.5	95.5%	S715-28D
21	37&38	100	237	9.6	1.15	0.0, 0.1, 0.0	78.8	0.6	0.6	93.7%	S715-26D
22	39&40	100	240	10.0	1.37	0.2, 0.0, 0.1	77.9	0.4	1.0	91.2%	S716-9D
25	41&42	100	244	6.0	0.84	0.3, 0.2, 0.2	76.4	0.4	0.7	93.8%	S716-30D

[1] % of charged sialic acid in the void volume

TABLE II. FERMENTOR PRODUCTION

Week No.	Batch No.	Broth Vol.	Wet Cell Wt.	Yield Step 5	Yield Final	Pyrogen Test	Sialic Acid	Protein	Nucleic Acid	Sephadex G-200[1]	Sample No.
21	F- 1	85	110	3.5	.436	0.0, 0.0, 0.0	76.8%	0.3%	0.4%		S715-19A
	F- 2	N O T	D E L I V E R E D								
22	F- 3	100	203	5.0	1.08	0.2, 0.1, 0.1	75.7	0.4	0.7	97.8	S715-28A
	F- 4	N O T	D E L I V E R E D								
22	F- 5	100	294	9.5	2.7	0.0, 0.0, 0.2	78.0	0.3	0.3	94.9	S716-24B
23	F- 6	100	472	15.5	5.04	0.0, 0.0, 0.0	78.8	0.4	0.4	90.1	S716-10C
	F- 7	100									
24	F- 8	100	250								
	F- 9 -- NOT DELIVERED			16.4	2.17	0.0, 0.0, 0.2	75.1	0.3	0.4	97.5	D716-19C
	F-10	100	261								
25	F-11	100	235								
	F-12	100	219	27.0	3.6	0.4, 0.0, 0.0	80.3	0.3	0.2	93.6	D717-7C
	F-13	100	263								
26	F-14	100	280	16.0	3.7	0.0, 0.5, 0.3	77.7	0.3	0.4	94.2	S717-13C
	F-15	100	271								

[1] Percent of charged sialic acid in void volume

FIGURE 1

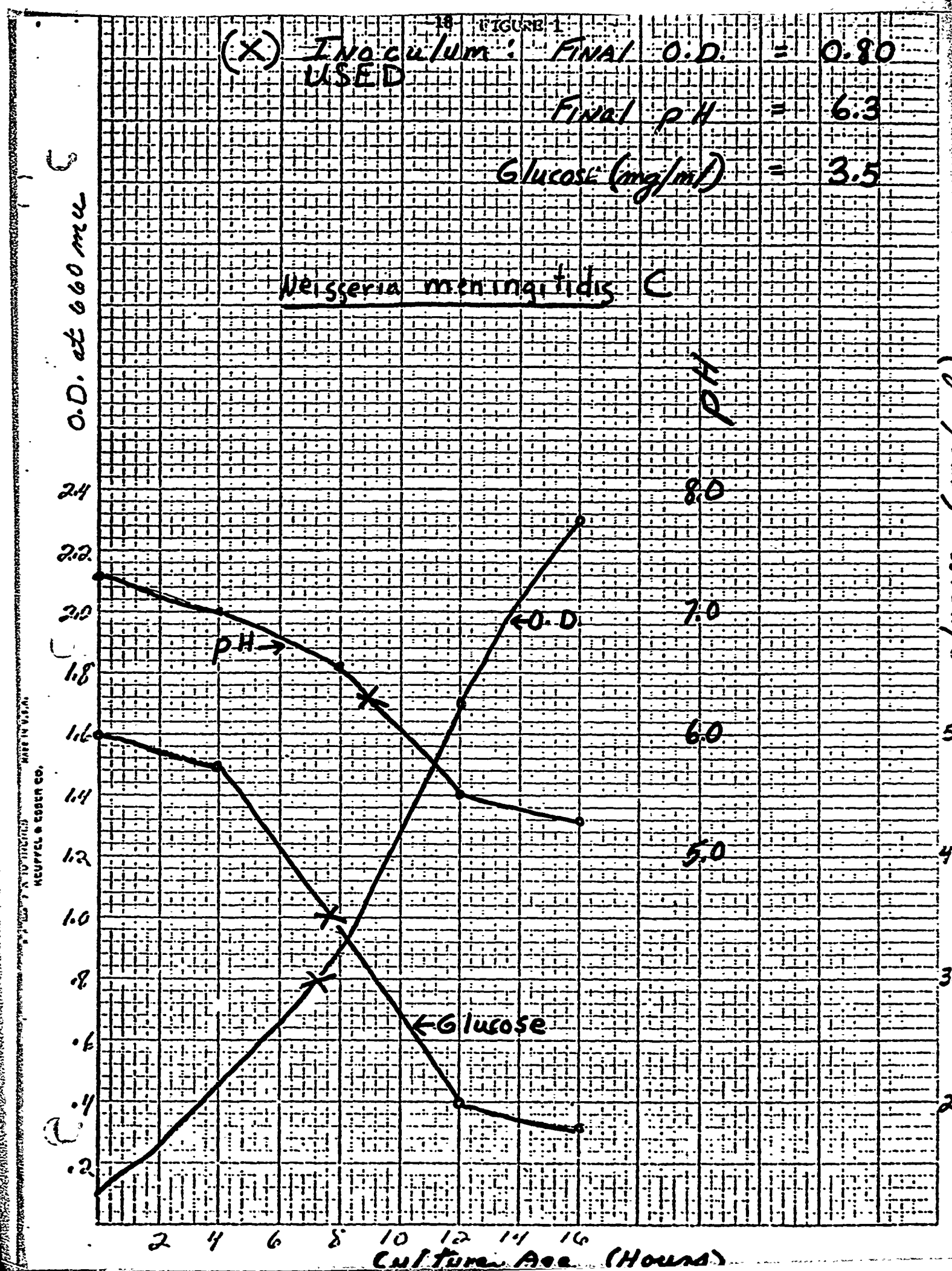

(X) Inoculum: Final O.D. = 0.80
Used Final pH = 6.3
Glucose (mg/ml) = 3.5
Neisseria meningitidis C
O.D. at 660 mμ
pH
Glucose (mg/ml)
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
8.0
7.0
6.0
5.0
5
4
3
2
pH →
← O.D
← Glucose
2 4 6 8 10 12 14 16
Culture Age (Hours)

TABLE III. DELIVERIES FOR FORMULATION

Delivery No.	Book No.	L-675,754-	Wt. Grams	Source/Batch	Delivery Date
1	S714-7F	-00G10	3.0	Shake-flask/ 18-23	4/7/71
2	S714-30F	-00G11	3.1	Shake-flask/ 24-28	5/12/71
3	S715-21F2	-00G12	3.1	Shake-flask/ 29-38	6/15/71
4	S716-4F	-00G13	3.4	Fermentor/ 6&7	6/22/71
5	S716-18F	-00G14	3.1	Fermentor/ 5-7	6/30/71
6	S717-9F	-00G15	2.2	Fermentor/ 5-8 & 10	7/7/71
7	S717-23F	-00G16	2.5	Shake-flask/ 18-28, 39-42	7/12/71
8	S718-20F	-00G17	3.5	Fermentor/ 11-13	7/14/71
9	S717-16F	-00G18	3.6	Fermentor 14&15	7/16/71

TABLE IV. Analyses of Deliveries for Formulation L-675,754-

Test \ Sample	00G10 S714-7F	00G11 S714-30F	00G12 S715-21-F-2	00G13 S716-4F	00G14 S716-18F	00G15 S717-9F	00G16 S717-23F	00G17 S718-20F	00G18 S717-16F	AVG.
Sialic Acid, %	75.1	75.6	75.1	78.9	77.1	77.1	78.6	77.4	77.9	76.9
Nucleic Acid, %	0.4	0.6	0.4	0.4	0.2	0.3	0.5	0.4	0.5	0.4
Protein, %	0.3	0.3	0.4	0.2	0.2	0.4	0.3	0.3	0.4	0.3
Gel Filtration M.W.,%(1)	99.6, 95.1	94.2	100	91.3	94.4	97.3	96.9	97.9	94.0	96
Moisture by TGA, % (2)	15.8	16.2	12.1	16.0	16.9	13.8	17.3	14.5	12.0	14.9
Acetone by GLC, %	0.06	0.02	0.05	0.1	0.03	0.02	0.26	0.01	0.5, 0.8	0.13
Calcium by AAS, % (3)	3.9+	3.39	4.20	4.55	4.29	4.40	3.89	4.18	4.15	4.20
Sulfated Ash, %	21.7	20.7	19.0	18.5	21.4	20.1	23.1	11.1	19.1	19.4
Total Acetyl, % (4)	17.5	14.5	15.9	17.5	17.8	15.1	12.9	15.6	13.3	15.5
Spectrographic Anal.										
Iron	<2 ppm	<2 ppm	<2 ppm	nil	nil	nil	nil	nil	<2 ppm	
Copper	<1 ppm	<1 ppm	<1 ppm	"	<1 ppm	<1 ppm	<1 ppm	nil	<1 ppm	
Sodium	10-50 ppm	<10 ppm	0.001-0.1%	<10 ppm	<10 ppm	<10 ppm	0.001-0.1%	<10 ppm	0.001-0.1%	
Magnesium	10-50 ppm	<2 ppm	<2 ppm	<2 ppm	<2 ppm	<2 ppm	<2 ppm	<2 ppm	<2 ppm	
Calcium	major ~3%	Imp. 0.005-0.1%	1-3%	0.001-0.1%	0.001-0.1%	0.01-1.0%	1-3%	1-3%	1-3%	
Boron	25-50 ppm	nil	nil	nil	nil	nil	nil	nil	nil	
Silicon	trace	"	"	"	"	"	"	"	"	

Notes: (1) Percent of charged sialic acid in void volume

(2) TGA = Thermogravimetric analysis

(3) AAS = Atomic absorption spectroscopy

(4) Total Acetyl, measuring N and O acetylation, is done by chromic acid oxidation.

TABLE V. SEPHAROSE 4B MOLECULAR WEIGHT DETERMINATIONS

Delivery No.	L# - 675,754-	K_{av}	M. W.	Type
1	00G10	.270	7.5×10^5	Shake Flask
2	00G11	.270	7.5×10^5	Shake Flask
3	00G12	.270	7.5×10^5	Shake Flask
4	00G13	.325	6.0×10^5	Fermentor
5	00G14	.310	6.7×10^5	Fermentor
6	00G15	.318	6.5×10^5	Fermentor
7	00G16	.270	7.5×10^5	Shake Flask
8	00G17	.290	7.2×10^5	Fermentor
9	00G18	.254	8.2×10^5	Fermentor
Squibb #	#9-"C"	.265	7.5×10^5	
Fermentor #3	S715-28A	.420	3.0×10^5	

TABLE VI. ANALYSES OF 80% ETHANOL PRECIPITATES

Week No.	Batch No.	Sample No.	Sialic Acid	Protein	Nucleic Acid
3	3 & 5	S711-22B1	62.0%	19.4%	1.9%
5	7 & 8	S712-2B	58.0	21.3	1.3
6	9 & 10	S712-9B	52.6	26.1	6.0
7	11 & 12	S712-17A	52.5	26.4	3.7
8	13	S712-23A	51.4	24.2	6.2
9	14 & 15	S713-2A	55.7	15.3	1.7
10	16 & 17	S713-9A	55.7	11.8	
11	18 & 19	S713-16A	48.0	15.6	2.0
21	F#1	S715-18A	48.0	24.7	4.6
22	F#3	S715-27A	46.6	26.5	5.6
22	F#5	S716-2A	56.5	15.0	2.5

TABLE VII. RESULTS OF SEVAGING

Week No.	Start		#1 Filtrate		#2 Filtrate		#3 Filtrate		#4 Filtrate	
	Sialic Acid	Protein	Sialic Acid	Protein	Sialic Acid	Protein	Sialic Acid	Protein	Sialic Acid	Protein
6	52.6%	26.1%	55.7%	25.4%	55.5%	23.7%	56.1%	22.1%	53.7%	21.6%
8	51.4%	24.2%		19.5		19.5		18.6		17.8
9	55.7	15.3	59.6	13.5	59.9	13.3	63.0	12.0	42.0	10.7
10	55.7	11.8	61.0	12.1	63.3	12.1	60.9	10.8	62.2	11.2

FIG. 2.

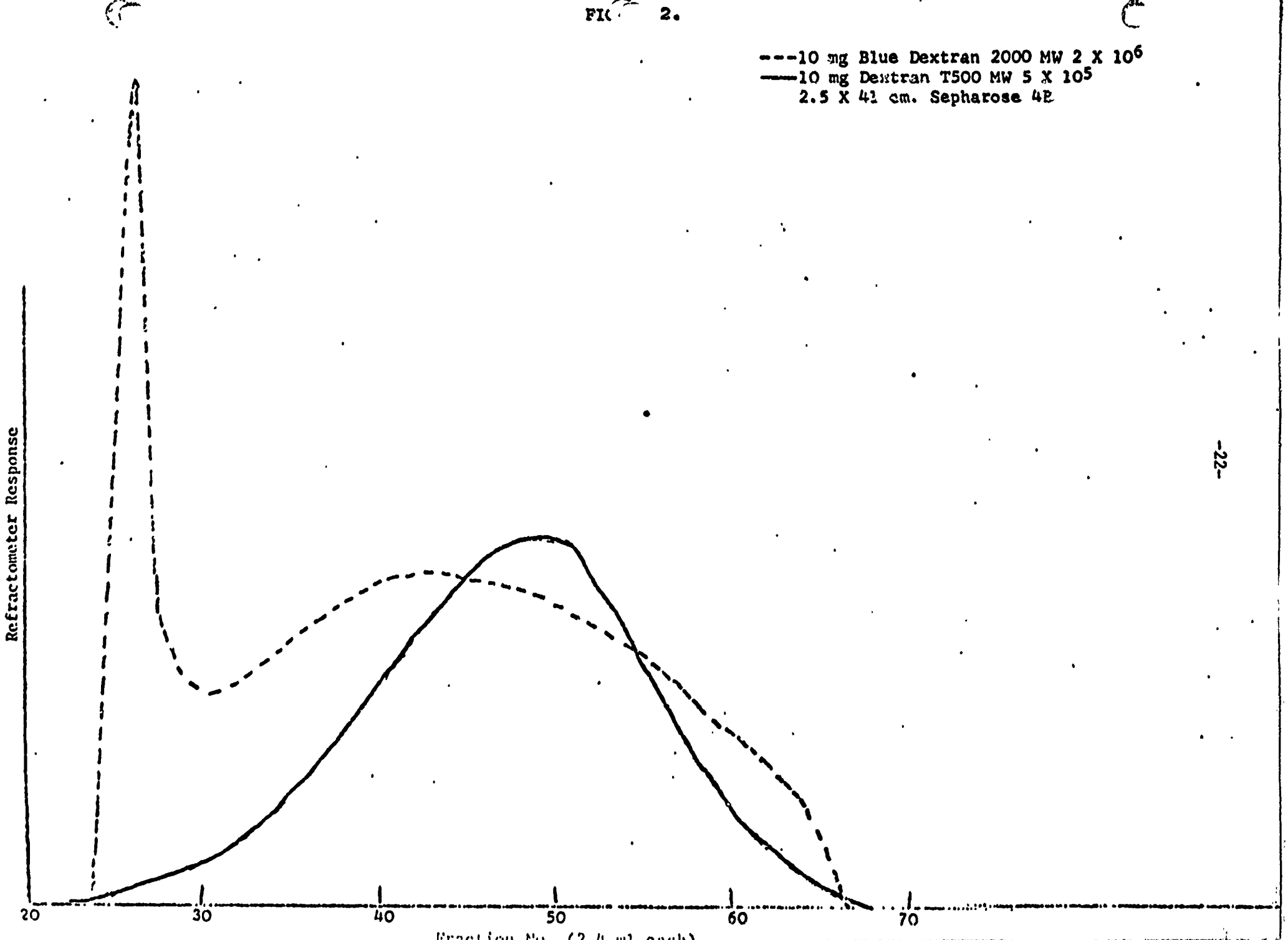

---10 mg Blue Dextran 2000 MW 2 X 10^6
——10 mg Dextran T500 MW 5 X 10^5
2.5 X 41 cm. Sepharose 4B
Refractometer Response
20
30
40
50
60
70

FIGURE 3.

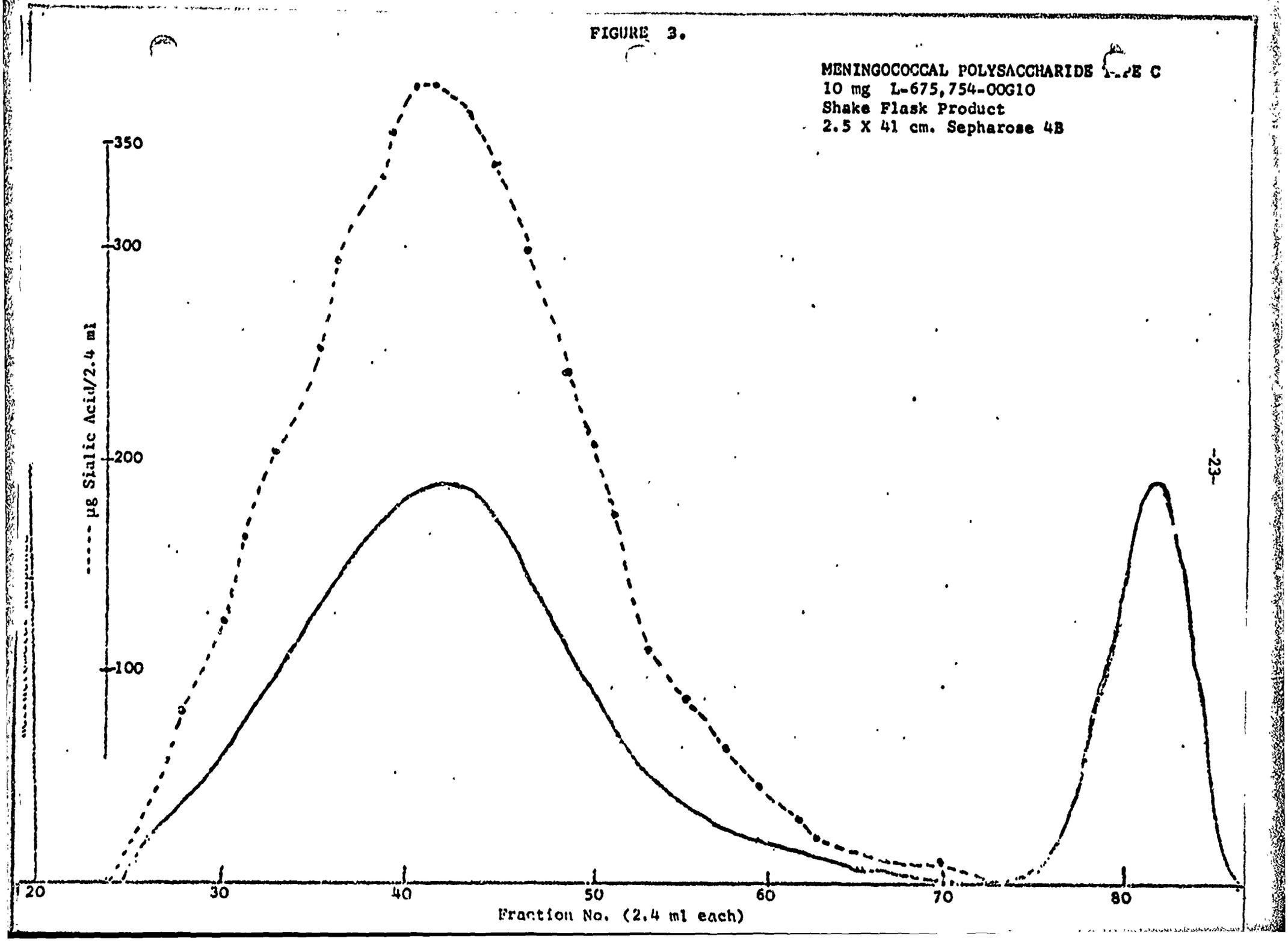

MENINGOCOCCAL POLYSACCHARIDE TYPE C
10 mg L-675,754-00G10
Shake Flask Product
2.5 X 41 cm. Sepharose 4B
µg Sialic Acid/2.4 ml
350
300
200
100
20
30
40
50
60
70
80
Fraction No. (2.4 ml each)

FIGURE 4.

MENINGOCOCCAL POLYSACCHARIDE TYPE C
10 mg L-675,754-00G15
Fermentor Product
2.5 X 41 cm. Sepharose 4B

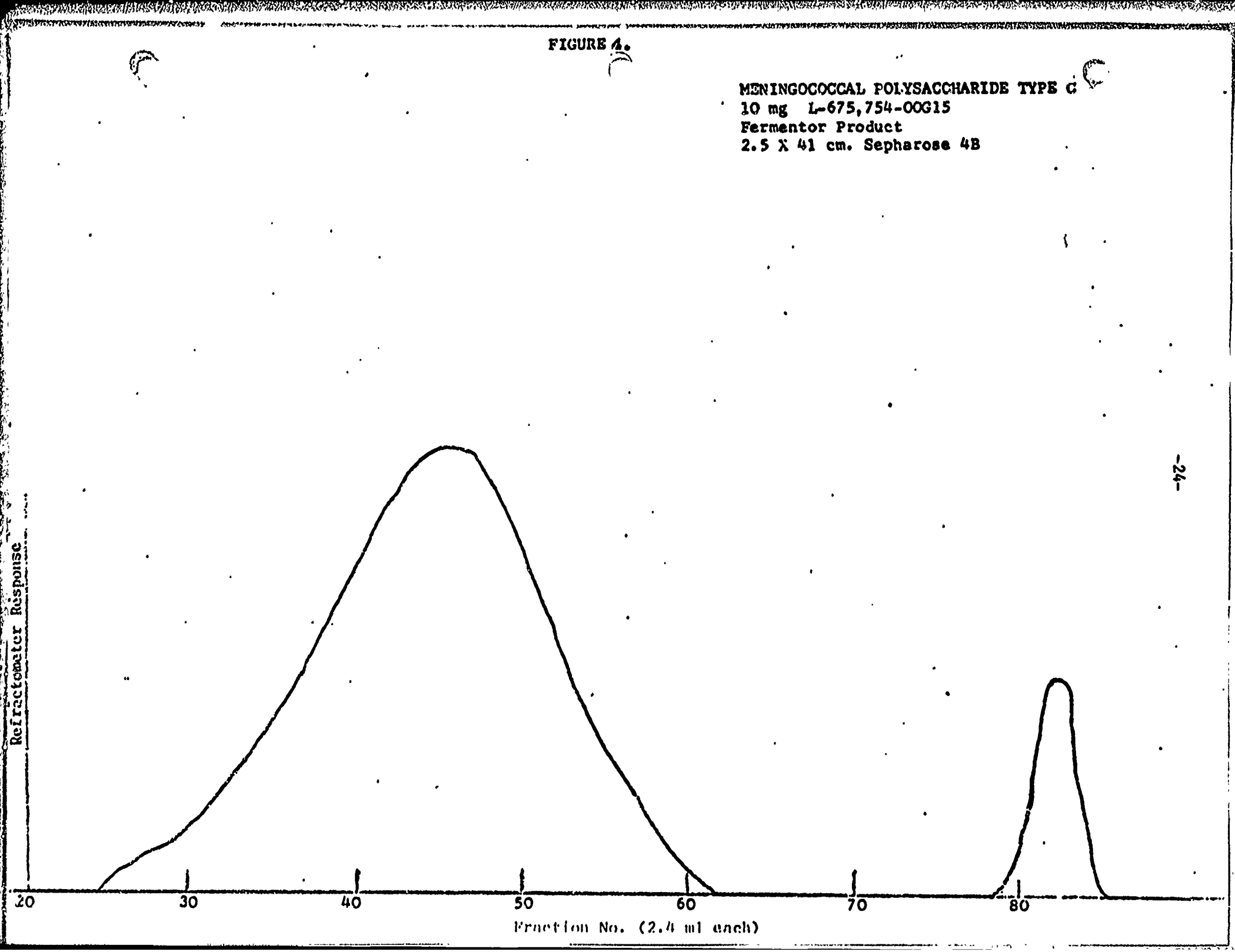


FIGURE 5.

MENINGOCOCCAL POLYSACCHARIDE TYPE C
10 mg L-675,754-00G16
Shake Flask Product
2.5 X 41 cm. Sepharose 4B

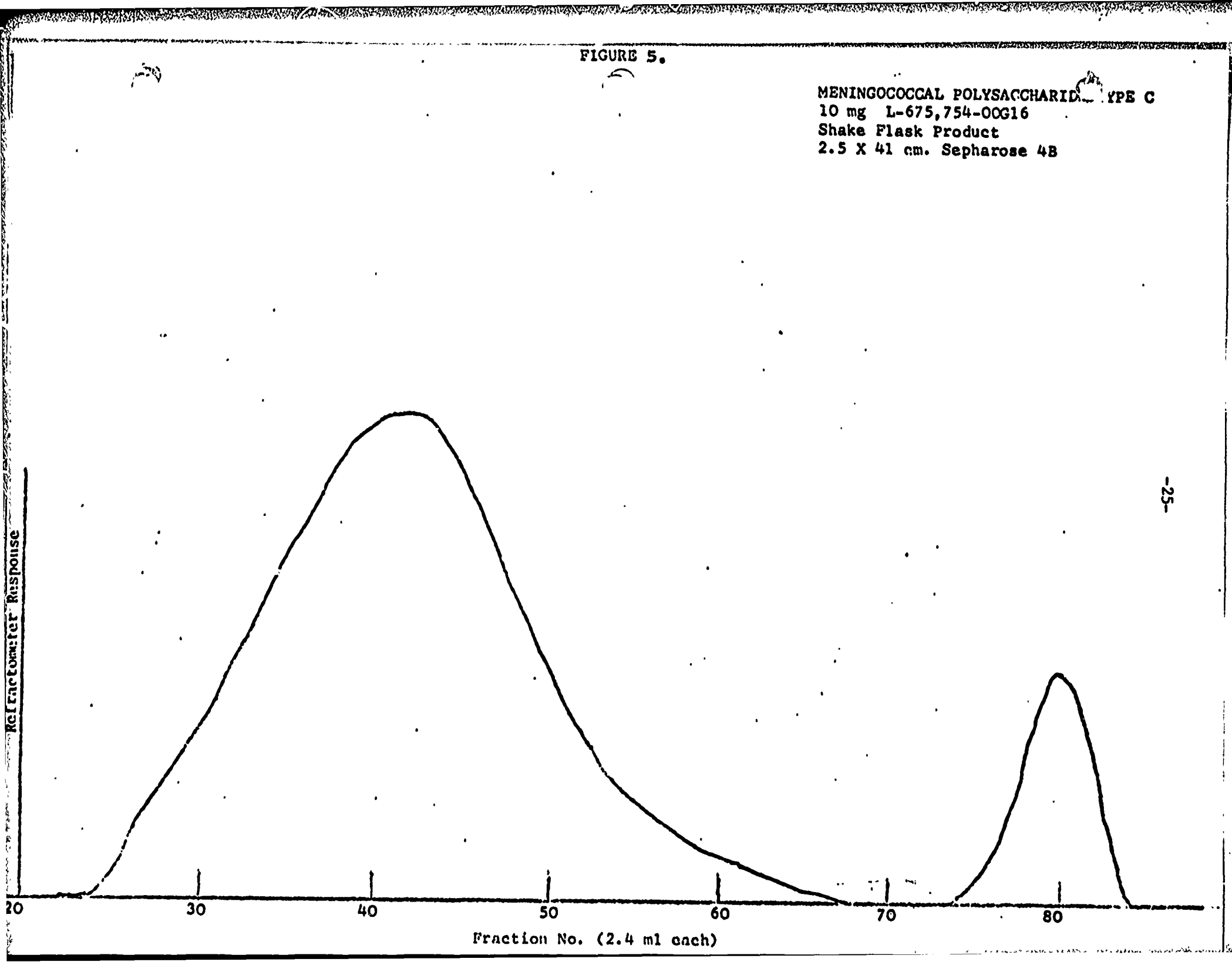
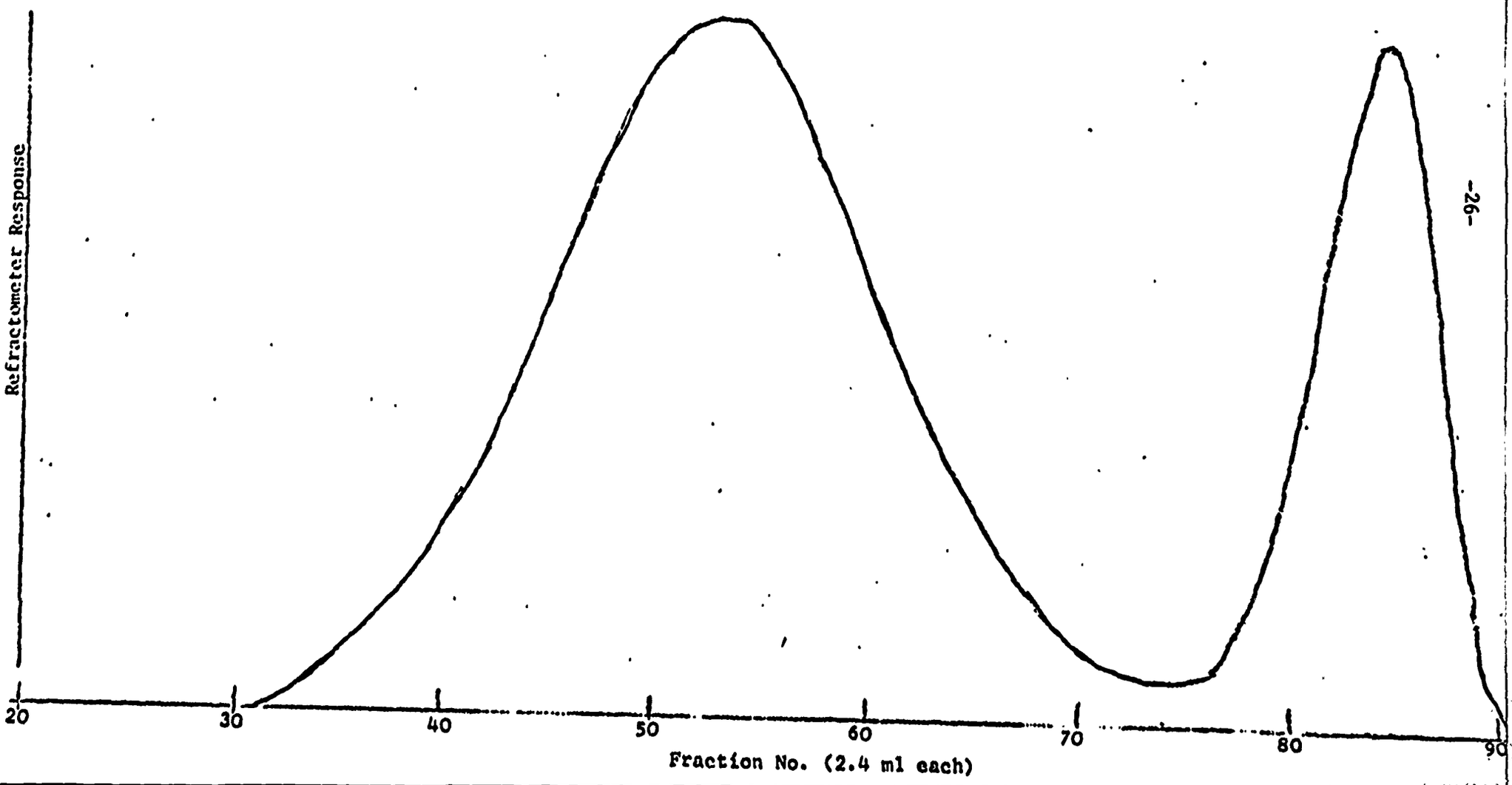


FIGURE 6.

MENINGOCOCCAL POLYSACCHARIDE T[illegible] C
1.08 g. S715-28A
Fermentor #3 Product
2.5 X 41 cm. Sepharose 4B



INDEX 1

MERCK SHARP & DOHME
MERCK SHARP & DOHME RESEARCH LABORATORIES
Division of Merck & Co., Inc.

BIOLOGICAL WORK ORDER

OB 264

BIOLOGICAL PRODUCTION, MSD		DATE OF REQUEST: 8/16/71	
FROM: A. P. Vella Virus & Cell Biology Research	DEPT. NO. 674	ACCTG CHARGES: (SPECIFY ACCT. NO. OR PROJECT NO.)	PROJECT 88-807-126

DESCRIPTION OF WORK REQUESTED: CONTROL

Please perform the following animal control tests as indicated below on Meningococcal polysaccharide type C, final container: Lot 430, C-A406, L-675-754, C0G18

A. Pyrogen test in rabbits (2 kg weight)

1. 3 rabbits each to receive 1.0 ml (2.5 mcg, intravenously) per 2 kg. of meningococcal polysaccharide type C, to be performed on each of 2 samples.

2. 3 rabbits to receive 1.0 ml of CM 66(distilled water) placebo intravenously.

3. Record rectal temperatures of all rabbits just prior to injection and 1, 2 and 3 hours post injection.

B. Safety test in mice (14-16 gm weight)

1. 20 mice to receive 0.5 ml (100 mcg, intraperitoneally) of meningococcal polysaccharide type C, each of two samples to be tested in 10 mice (20 total).

2. 20 mice to receive 0.5 ml of CM 66 (distilled water) placebo intraperitoneally.

3. Observe and weigh all animals daily for 7 days; record weights and submit on assay report.

C. Safety test in guinea pigs (350 gm weight)

1. 4 guinea pigs to receive 1.0 ml (500 mcg, intraperitoneally) of meningococcal polysaccharide type C, each of two samples to be tested in 2 guinea pigs (4 total).

2. 3 guinea pigs to receive 1.0 ml of 4.6% saline placebo intraperitoneally.

3. Observe, weigh and check rectal temperatures for all animals daily for 7 days. Record temperatures and weights and submit on assay report.

D. Please submit results to Dr. Vella in the form of written assay reports. A Certificate of Analysis is required also.

IF ESTIMATED COST IS LESS THAN $_____ THIS REQUEST IS VALID WITHOUT FURTHER APPROVAL.	ORIGINATED BY: T. J. Kucharski
	ACCEPTED BY BIOLOGICAL PRODUCTION:
ESTIMATED COST:	ESTIMATE APPROVED BY:

BIOLOGICAL WORK ORDER NO. [illegible]
TO TRANSMIT RESULTS TO CONTROL OFFICE
COPIES OF FINAL REPORT WILL BE PREPARED

ROUTING:

1. ORIGINATOR WILL FORWARD ALL COPIES TO DIRECTOR, BIOLOGICAL PRODUCTION.
2. DIRECTOR, BIOLOGICAL PRODUCTION WILL SUPPLY ESTIMATE AND ACCEPTANCE.
3. IF ESTIMATE IS LESS THAN THE STIPULATED AUTHORIZATION, DISTRIBUTE COPIES AS FOLLOWS:
   2 COPIES TO ORIGINATOR OR ORIGINATED BY DEPARTMENT OF [illegible] WILL BE FORWARDED TO [illegible]
   1 COPY TO PRODUCTION CONTROL
   3 COPIES TO BE RETAINED BY DIRECTOR, BIOLOGICAL PRODUCTION.
4. IF ESTIMATE EXCEEDS STIPULATED AUTHORIZATION RETURN COMPLETE SET TO ORIGINATOR FOR NECESSARY APPROVALS. WHEN THESE ARE OBTAINED, SET IS RETURNED TO DIRECTOR, BIOLOGICAL PRODUCTION FOR DISTRIBUTION AS STIPULATED IN ITEM 3.

INDEX 2

BIOLOGICAL WORK ORDER

...CK SHARP & DOHME
...CK SHARP & DOHME RESEARCH LABORATORIES
...ision of Merck & Co., Inc.

OH 294

BIOLOGICAL PRODUCTION, MS&D		DATE OF REQUEST: 8/20/71
...n: Dr. Vella Virus & Cell Biology Research	DEPT. No. 874	ACCTG CHARGES: (SPECIFY ACCT. NO. OR PROJECT NO.) 88-807-126

...SCRIPTION OF WORK REQUESTED:

CONTROL

Please perform a safety test in mice as indicated below on each of the 8 listed Meningococcal polysaccharide type C, final container, vaccine lots.

A. Safety test in mic... ...-16 gm weight)

1. 20 mice for each vaccine lot (160 total) to receive 0.5 ml (100 mcg, intraperitoneally) of Meningococcal type C polysaccharide; there will be two samples for each lot (10 mice/sample, 20 total/vaccine).

2. 20 mice to receive 0.5 ml of CM 66 (distilled water) placebo intraperitoneally. These 20 mice will be suitable controls for the entire test as long as all animals are placed on test at the same time.

3. Observe and weigh all animals daily for 7 days; record weights and submit on assay report.

Vaccine lots

1. Lot 419, C-A258	2. Lot 420, C-A259	3. Lot 421, C-A260
4. Lot 422, C-A261	5. Lot 423, C-A262	6. Lot 424, C-A263
7. Lot 438, C-A406	8. Lot 419, C-A258-1,2	

Please submit results to Dr. Vella in the form of written assay reports; a Certificate of Analysis is required also.

cc: Newman
Weber
Mitchell
Daniel
Vella

BIOLOGICAL WORK ORDER NO. 1560
REPORT RESULTS TO CONTROL OFFICE
CERTIFICATE OF ANALYSIS WILL BE PREPARED BY BIOLOGICAL CONTROL OFFICE.

IF ESTIMATED COST IS LESS THAN $_____ THIS REQUEST IS VALID WITHOUT FURTHER APPROVAL

ORIGINATED BY: Ronald E. Mitchell

ACCEPTED BY BIOLOGICAL PRODUCTION:

ESTIMATED COST:

ESTIMATE APPROVED BY:

...ING:

1. ORIGINATOR WILL FORWARD ALL COPIES TO DIRECTOR BIOLOGICAL PRODUCTION.
2. DIRECTOR, BIOLOGICAL PRODUCTION WILL SUPPLY ESTIMATE AND ACCEPTANCE.

IF ESTIMATE IS LESS THAN THE STIPULATED AUTHORIZATION ORDER 01. COPIES AS FOLLOWS:

1 COPY TO PRODUCTION CONTROL
3 COPIES TO BE RETAINED BY DIRECTOR, BIOLOGICAL PRODUCTION.

4. IF ESTIMATE EXCEEDS STIPULATED AUTHORIZATION RETURN COMPLETE SET TO ORIGINATOR FOR NECESSARY APPROVALS. WHEN THESE ARE OBTAINED, SET IS RE-

Index 3

Sedimentation Analysis

The sedimentation analysis of the polysaccharide was performed in a Spinco Model E analytical centrifuge under the following conditions:

1. polysaccharide concentration* at 0.5 mg/ml
2. solution - 0.85% NaCl
3. rpm - 48,000 ($W^2 = 2.54 \times 10^7$)
4. cell - 30 mm
5. rotor - Model E
6. temperature - 20°C
7. Time - 1.5 hours

Materials with sedimentation coefficients about 4.5S normally have molecular weight of approximately 100,000; this can be influenced by shape and degree of solvation.

*Note: lyophilized, final container vaccine (diluted to 500mcg/ml) was dialyzed against CM258 (pyrogen-free, 0.85% saline); 2.0 ml of diluted vaccine against one liter of CM258 for 24 hours.

Index 4

Glassware, rubber and metal tubing and filter holder preparation

A. All containers (4 liter bottles with rubber tubing and stainless steel connecting pieces) were prepared in the Merck Sharp & Dohme Biological Production area (Mr. E. Lewis, West Point, Pa.) according to a standard procedure utilized for preparing such materials to eliminate possible extraneous sources of pyrogenicity. Pyrogen-free water rinses and dry wall heating of glassware were performed while tubing and metal connections were rinsed with pyrogen-free water including a step utilizing water at 180°F and subsequently autoclaved.

B. All other glassware, such as volumetric flasks and pipettes and beakers were prepared in a similar manner in the Department of Virus and Cell Biology Research, MSDRL, West Point, Pa.

C. New Millipore filter holders (142 mm) were flushed initially with four liters of boiling, pyrogen-free, distilled water. Prior to each filtration of polysaccharide solution, the pre-filter and filter were flushed with 500 to 600 ml of pyrogen-free polysaccharide diluent (11.25% saline X CM27). After filtration, the filter units were flushed with three to four liters of pyrogen-free water (CM66) and autoclaved immediately.

PREPARATION OF PYROGEN-FREE EQUIPMENT

Glassware is washed either on the Better Built Hydromatic or Turbomatic washer and subjected to the treatment described below.

Hydromatic Washer

1. Pre-rinse - recirculated water
2. Wash cycle - recirculated detergent charged water, heated to 160°
3. Rinse - recirculated tap water from rinse tank, heated to 160°
4. Rinse - direct hot tap water, 150°
5. Rinse - pyrogen-free distilled water, 180°

Turbomatic Washer

1. Pre-rinse - recirculated water
2. Wash cycle - recirculated hot tap water charged with detergent, Aura, Temperature 160°
3. Rinse - recirculated hot tap water, Temperature 160°
4. Rinse - recirculated pyrogen-free water, Temperature 180°
5. Rinse - direct pyrogen-free rinse, Temperature 180°

Rubber tubing and machine components are subjected to the treatment described below.

Tumbler Washer

1. Tubing and components placed in barrel of tumbler and covered with hot tap water.
2. Boil tubing for full five (5) minutes. (No detergent used.)
3. Rinse - Pyrogen-free distilled water, 180° temperature for five (5) minutes while tumbling.
4. Rinse - Pyrogen-free distilled water, 180° temperature for five (5) minutes while tumbling.
5. Drain and jog to eliminate excess water in tubing.

Machines Assembly

1. Cut tubing to proper lengths.
2. Inspect stoppers, S.S. connectors and S.S. 'L's.
3. Plug air filter and insert in tubing; connect to SS. 'L'.
4. Assemble tubing, stoppers, and S.S. components.
5. Rinse all parts with pyrogen-free distilled water.
6. Wrap exterior ends of machines using gauze, paper envelopes, and Twistems.
7. Place Diack control on S.S. 'L'.

Assembly of Machine and Bottle Units

1. Inspect bottles and machines.
2. Add approximately 10-25 cc pyrogen-free distilled water to bottle.
3. Insert machine in bottle.
4. Adjust 'C' clamp to hold stopper/machine firmly in bottle.
5. Cover stopper and neck of bottle with aluminum foil.

Sterilization is by autoclave at 250°F. for a minimum of 30 minutes.

8/23/71

Index 5

Polysaccharide Diluent

A sodium chloride solution of 11.25% (XCM-27) is required so that rehydration of the polysaccharide (1:12.5x dilution) to 100 mcg/ml will yield approximately 9.0 mg NaCl/ml. In order to compensate for the 12.0% value obtained for the XCM-27, 70.0 ml of pyrogen-free, distilled water were added to each 1000.0 ml of 12.0% saline solution to yield a final concentration of 11.25%.

Index 6

Microbial Sterility

A. Post-filtration, bulk pool

1. Three to 5.0 ml of the polysaccharide concentrate (1.25 mg/ml) are inoculated into each of four bottles of fluid thioglycollate medium (150 ml medium per bottle) and incubated at 32°C for 14 days. The bottles are examined two to three times prior to the final reading for evidence of microbial contamination.

2. Three to 5.0 ml of the polysaccharide concentrate (1.25 mg/ml) are inoculated into each of four bottles of trypticase soy medium (150 ml medium per bottle) and incubated at 20-25°C for 14 days. The bottles are examined two to three times prior to the final reading for evidence of microbial contamination.

B. Lyophilized, final product, lot 438, C-A406

1. Six (6), 8 and 6 vials were obtained from the beginning, middle and end of the filling operation respectively. One (1.0 ml) was removed from each of the 20 vials after reconstitution with 25.0 ml distilled water to give a polysaccharide concentration of 100.0 mcg/ml and inoculated into 20 bottles of fluid thioglycollate medium (150 ml medium per bottle), incubated and observed as indicated as in A. 1. above. Additionally 1.0 ml was removed from each container and inoculated into 20 bottles of trypticase soy medium (150 ml medium per bottle), incubated and observed as indicated in A. 2. above.

MERCK SHARP & DOHME
MERCK SHARP & DOHME RESEARCH LABORATORIES
Division of Merck & Co., Inc.

BIOLOGICAL WORK ORDER

OD 284

TO: BIOLOGICAL PRODUCTION, MS&D		DATE OF REQUEST: 8/16/71
FROM: Dr. P. Vella Virus & Cell Biology Research	DEPT. No. 874	ACCT'G CHARGES: (SPECIFY ACCT. NO. OR PROJECT NO.) 88-807-125

DESCRIPTION OF WORK REQUESTED:

CONTROL

Sterility Tests

Please check the indicated number of final containers of the indicated lot of Meningococcal polysaccharide for sterility in trypticase soy and fluid thioglycollate medium according to the latest Public Health Service Regulations.

1. Lot 438, C-A405 -20 final containers (labeled 1, 2 and 3)

Add 1.0 ml of polysaccharide solution per bottle of testing medium. The fluid thioglycollate medium should be incubated at 32° for 14 days while the trypticase soy medium should be incubated at 20 - 25° C for 14 days. Please submit results to Dr. Vella in the form of written assay reports. A Certificate of Analysis is required also.

BIOLOGICAL WORK ORDER NO. 1567
REPORT RESULTS TO CONTROL OFFICE
CERTIFICATE OF ANALYSIS WILL BE PREPARED BY BIOLOGICAL CONTROL OFFICE.

IF ESTIMATED COST IS LESS THAN $_____ THIS REQUEST IS VALID WITHOUT FURTHER APPROVAL.	ORIGINATED BY: [signature]
	ACCEPTED BY BIOLOGICAL PRODUCTION:
ESTIMATED COST:	ESTIMATE APPROVED BY:

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4. IF ESTIMATE EXCEEDS STIPULATED AUTHORIZATION RETURN COMPLETE SET TO ORIGINATOR FOR NECESSARY APPROVALS. WHEN THESE ARE OBTAINED, SET IS RETURNED TO DIRECTOR, BIOLOGICAL PRODUCTION FOR DISTRIBUTION AS OUTLINED IN PARAGRAPH 3.

Index 7

BIOLOGICAL TESTING PROCEDURE

PHARMACEUTICAL LABORATORY

I. SAMPLES

Final container samples of completed product shall be tested. Individual samples having a low net weight shall be pooled at the time of testing in order to attain an adequate sample weight.

II. TEST PROCEDURE

A minimum of 100mg. of dried product obtained from one or more final containers shall be used. The test shall consist of determining the weight loss of the sample(s) due to drying for 72 hours under vacuum over phosphorous pentoxide.

A. Recommended materials and equipment:

1. Numbered, low form, flat-bottom weighing dishes with well-ground glass stoppers
2. Vacuum desiccator equipped with a vacuum pump and an uncalibrated manometer
3. Balance capable of accurate readability to 0.1 mg.
4. Low humidity room equipped with hygrometer and maintained at a relative humidity of 0 - 10%. All operations are performed in this room.
5. Desiccator jar equipped with silica gel drying agent with indicator

B. Method:

1. Thoroughly cleaned weighing dishes shall be dried by hand and stored in the low humidity room for 24 hours prior to use. NOTE: All equipment is kept in the low humidity room at all times.
2. The weighing dishes shall be weighed as rapidly as possible. All manipulations of weighing dishes shall be made with tongs or while wearing gloves.

B. Method (Cont'd)

3. The vacuum in the sample bottle shall be released by allowing dry air to enter slowly through a needle inserted in the stopper.

4. The stopper shall be removed and the sample plug broken up with a spatula.

5. The sample shall be rapidly transferred to a previously weighed and marked weighing dish and covered with its lid.

6. After all transfers have been completed, the weighing dishes with their contents shall be weighed immediately.

7. The samples and the weighing dishes with the lids set perpendicular to the normal position shall be placed in the vacuum desiccator. The pressure shall be reduced as indicated by the manometer and the reduced pressure maintained by sealing the desiccator.

8. After 72 hours of drying time, dry air is allowed to continually bleed into the desiccator until the pressure inside has been equalized with the atmosphere.

9. The lids shall be immediately replaced in the normal closed position.

10. The weighing dishes containing the samples shall be removed individually from the desiccator and weighed as rapidly as possible.

11. All samples are to be tested in duplicate and run with a standard in each desiccator.

C. Calculations:

1. Record weights in Steps 2, 6, and 10.

2. Weight in Step 2 equals tare weight of dish.

3. (Weight in Step 6) minus (weight in Step 1) equals weight of sample before assay.

4. (Weight in Step 6) minus (weight in Step 10) equals weight equivalent to residual moisture of sample.

5. (Weight equivalent to moisture) divided by (weight of sample) times 100 equals the percentage of residual moisture.

III. REQUIREMENTS

A. Final container samples of completed product shall be used. Sample weight shall be 100 to 1,000 mg.

B. If only one sample is tested, the serial or subserial is unsatisfactory if the moisture content exceeds 2.0% for vaccines only.

C. If any determination is greater than 2.0%, a repeat test must be done.

D. The repeat test will consist of four determinations run in duplicate on two separate days. The lot will only be considered satisfactory if no values on repeat determinations are above the 2.0% limit and the average of all six determinations is below the 2.0% limit.

E. In the event that one of the two determinations is over 2.0%, a retest will be done.

The repeat test will consist of two determinations. The lot will only be considered satisfactory if no values on repeat determinations are above the 2.0% limit and the average of all four determinations is below the 2.0% limit.

NOTE:

'Dornovac' is always assayed on the Moisture Monitor.

1. Only the content of one vial is used.

2. The sample is weighed on the Microbalance (accuracy to 0.01 mg.).

3. Sample is tested in triplicate.

4. Calculation:

$$\frac{\text{Moisture Monitor reading in mg.}}{\text{Sample weight in mg.}} \times 100\% = \% \text{ moisture}$$

Index 8

Determination of Chloride in RCM6 or RCM8

(based on a modification of Swain, J.S., Chemistry and Industry, 1956, pp. 418-420)

I. Preparation of Standards

A. Weigh 2.103 gms of C.P. KCl into a 1-liter volumetric flask, dissolve and dilute to mark with RCM2, thus preparing a solution containing 1000 mcg/ml Cl^-. Dilute appropriately with RCM2, using volumetric pipettes and flasks, to obtain standard solutions containing 20, 22.5, 25, 27.5 and 30 mcg/ml Cl^-.

II. Preparation of Samples

A. Using volumetric pipettes and flasks, dilute 5.0 ml of samples to 1000 ml with RCM2. Since RCM6 and RCM8 each theoretically contains 5368 mcg/ml Cl^-, this will give a solution of the sample containing approximately 26.84 mcg/ml Cl^-.

III. Chemicals Used

Acetone, ACS Reagent Grade
Methanol, ACS Reagent Grade
Ferric Alum, C.P.
Nitric Acid, Conc., C.P.
Mercuric Thiocyanate (may be Fisher Certified or made from C.P. $Hg(NO_3)_2$ and C.P. KSCN)
RCM2 (glass distilled water)

IV. Preparation of Reagents - for Beckman DU Procedure

A. $Hg(SCN)_2$ - Dissolve 300 mgs of $Hg(SCN)_2$ in 100 ml methanol. Filter if necessary.

B. Ferric Alum - Dissolve 6.0 gms Ferric Alum in 100 ml 6N nitric acid (39 ml conc. nitric acid plus 61 ml RCM2). Filter thru fine sintered glass.

V. Procedure - Beckman DU

A. In a series of 6 x 7/8" test tubes, pipette 5.0 ml of RCM2 (for 0 blank), standard solutions and replicate sample solutions. To each tube add 5.0 ml of acetone. Mix. Add 1.0 ml ferric alum reagent. Mix. Add 1.0 ml $Hg(SCN)_2$ reagent. Mix, let stand 15 minutes and read λ470. Plot data and calculate concentrations in the usual way.

VI. Preparation of Reagents - for Spectronic 20 Procedure

A. $Hg(SCN)_2$ - Dilute one volume of the $Hg(SCN)_2$ solution described above with 9 volumes of acetone.

B. Ferric Alum - Dilute 2 volumes of the ferric alum reagent described above with 3 volumes of RCM2.

Index 8 (cont'd.)

VII. Procedure - Spectronic 20

A. In a series of 6 x 7/8" test tubes, pipette 5.0 ml of RCM2 (for 0 blank), standard solutions and replicate sample solutions. Add 5.0 ml of ferric alum reagent, mix. Add 10.0 ml $Hg(SCN)_2$ reagent, mix. Let stand 15 min. and read at λ 470. Plot data and calculate concentrations in the usual way.

VIII. Notes

A. The optical density scale of the Spectronic 20 is so constructed that readings can be made much more accurately in the 0.05-0.3 region than in the 0.3 to 1.0 region. This is not true to a significant degree with the Beckman DU. Accordingly, the procedure used with the Beckman DU has been modified for use with the Spectronic 20. The latter procedure has only 1/2 the sensitivity of the former but is quite usable.

B. The color developed in this procedure is quite stable; there is a loss of approximately 1% in a 45-minute period following the usual reading time.

C. This method is not affected by even large concentrations of phosphate.

IX. Calculations

A. For physiological saline (RCM6) and phosphate buffered physiological saline, use the following:

1. Average reading of sample x 200 (dilution) x 27.5 = mcg Cl^- in sample, average reading of 27.5 standard.

2. Cl^- = 60.8% of the weight of the NaCl molecule.

Index 9

Meningococcal (type C) Hemagglutination Procedure

Reagents

Human O, Rh negative red blood cells (Behringwerke diagnostics - type-a-cells 35 kit)
RCM8 - store at 4°C
Bovine serum albumin 10% (sterile) store at 4°C
Meningococcal antigen (polysaccharide) stored at -70°C; thawed at 37°C
Cooke Microtiter plates and seals
Microtiter loops (0.05 ml)

Procedure

1. Wash human O negative red blood cells three (3) times in RCM8.
2. Resuspend after final wash to 2%.
3. Add appropriate amount of antigen (determined by grid titration) to the cells.
4. Mix thoroughly, incubate 30 min. (or longer) in 37°C water bath, mix and invert every 5-7 minutes.
5. Wash RBC-antigen three (3) times in RCM8 and resuspend to a 0.5% suspension in RCM8.
6. Add bovine serum albumin to a final concentration of 0.5% w/v. (1 ml of 10% BSA +19 ml rbc-antigen).

   a. Defrost sera and heat inactivate; 56°C for 30 minutes.

   b. Prepared serial 2-fold dilutions (1:2 → 1:2048) in RCM8.

   c. 0.05 ml RBC-antigen suspension is added to each well.

   d. Mix gently.

   e. Incubate plates 2 hrs. at R.T. and then read for HA titer.

Merck & Co., Inc.

BIOLOGICAL PRODUCTION, MSRD		DATE OF REQUEST: 5/24/71	
Dr. Vella & Cell Biology Research	DEPT. NO. 874	ACCT'G CHARGES: (SPECIFY ACCT. NO. OR PROJECT NO.)	88-807-326

DESCRIPTION OF WORK REQUESTED:

PRODUCTION

A. The following sterile solutions of Neisseria meningitidis (Meningococcal) polysaccharide type C at 1.25 mg/ml are to be dispensed as indicated below into vials suitable for subsequent lyophilization and will be available on the indicated dates. (See attached sheet)

B. Scheduling

1. Please notify Dr. Vella of the filling dates so that the polysaccharide powder for each lot may be rehydrated and Millipore filtered on the same day.

2. In addition to the filling schedule, please notify Dr. Vella of the lyophilization schedule so that he may be present at the initiation of each operation.

C. Filling

Dispense 2.0 ml of each polysaccharide lot into at least 1100 vials capable of holding 25.0 ml of diluent.

NOTE: The rehydrated polysaccharide should be stored at -50° C.

D. Lyophilization

1. Please schedule the above vaccine lots for lyophilization according to the procedure received from the Walter Reed Army Institute of Research.

2. The lyophilized material should be returned to Dr. Vella, 26-[illegible], and he will remove the appropriate samples for control testing.

E. Records

Appropriate copies of notebook records with dates, procedures, etc. used for the filling and lyophilization of these lots are requested

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TABLE VIII

Neisseria meningitidis, type C polysaccharide, final container assays

Vaccine Designation	Amount Weighed Gm.	Diluent Vol. ml	NaCl %	Pyrogen resp. max. rise, °C/rabbit (2 vials)	Sterility TSB* # contam./# inoc.	Sterility Thio* # inoc.	Animal Safety Tests** Guinea Pig 7d wt.gain gm B	E	P	Mouse 7d wt.gain gm B	E	P	Sedimentation+ Analysis	Moisture Content,% (1-2 vials)	Sialic Acid %	Identity (HA assay)
419, C-A258 L675,754-00C10,S714-7F	2.9936	2395	0.96	0.0, 0.1, 0.2 0.0, 0.0, 0.1	0/20	0/20	17.5	22.5	21.7	6.53	5.62	9.0	4.4S, 1 comp.	0.53	75.0	Satisfactory
420, C-A259 L675,754-00C11,S714-30F	3.0	2400	0.98	0.1, 0.0, 0.0 0.3, 0.0, 0.1	0/20	0/20	35.0	42.5	31.7	6.58	6.38	9.0	4.5S, 1 comp.	0.27, 0.29	76.0	Satisfactory
421, C-A260 L675,754-00C12,S715-21F2	3.0963	2477	0.88	0.4, 0.2, .0 0.3, 0.2, 0.0	0/20	0/20	42.5	25.0	43.3	6.45	6.93	9.0	4.1S, 1 comp.	0.26, 0.16	75.5	Satisfactory
422,C-A261 L675,754-00C13,S716-4F	3.3274	2622	0.90	0.1, 0.2, 0.4 0.3, 0.0, 0.1	0/20	0/20	37.5	40.0	28.3	7.0	6.97	9.0	4.1S, 1 comp.	0.49, 0.76	80.0	Satisfactory
423, C-A262 L675,754-00C14,S716-18F	3.0412	2433	0.99	0.0, 0.1, 0.0 0.2, 0.0, 0.2	0/20	0/20	25.0	47.5	43.4	7.43	7.38	9.0	4.2S, 1 comp.	0.52, 0.42	79.5	Satisfactory
424, C-A263 L675,754-00C15,S717-9F	2.1479	1680	0.90	0.2, 0.0, 0.0 0.5, 0.0, 0.0	0/20	0/20	57.5	45.0	41.7	6.28	7.12	9.0	4.1S, 1 comp.	0.41, 0.14	75.5	Satisfactory
426, C-A265 L675,754-00C17,S718-20F	3.6576	2920	0.93	0.4, 0.2, 0.0 0.5, 0.2, 0.0	0/20	0/20	57.5	20.0	45.0	7.49	6.69	8.06	3.9S, 1 comp.	0.54, 0.36	80.5	Satisfactory
433, C-A406 L675,754-00C18,S717-16F	3.1747	2537	0.88	0.0, 0.0, 0.1 0.0, 0.1, 0.1	0/20	0/20	62.5	47.5	65.0	7.65	6.32	9.0	4.6S, 1 comp.	0.67, 0.59	85.5	Satisfactory

* trypticase soy broth and fluid thioglycollate medium incubated at 20-25°C and 32°C, respectively, for 14 days

** samples taken from the beginning and end of the final container dispensing operation, each were injected into 2 guinea pigs (4 total) and 10 mice (20 total); the placebo was injected into a total of 3 guinea pigs and 20 mice.

+ Spinco model E ultracentrifuge

TABLE IX

Neisseria meningitidis, type C polysaccharide

Initial Batch Pyrogen and Sedimentation Analysis Assays

Vaccine Designation	Component Batches, Designation	Pyrogen Response, Max. Rise, °C/Rabbit	Sedimentation Analysis
Lot 419	S713-20A	0.0, 0.0, 0.2	4.4S, 1 component
S714-7F	S713-26A	0.3, 0.4, 0.0	4.2S, 1 component
	S714-1D	0.2, 0.0, 0.3	4.6S, 1 component
Lot 420	S714-7C	0.4, 0.0, 0.0	4.6S, 1 component
S714-30F	S714-15C	0.1, 0.0, 0.1	4.1S, 1 component
	S714-22C	0.2, 0.3, 0.4	4.5S, 1 component
Lot 421	S715-7B	0.0, 0.1, 0.1	4.6S, 1 component
S715-21F2	S715-6C	0.2, 0.3, 0.2	5.1S, 1 component
	S715-12C	0.1, 0.2, 0.0	4.1S, 1 component
	S715-28D	0.1, 0.2, 0.1	3.9S, 1 component
	S715-26D	0.0, 0.1, 0.0	4.4S, 1 component
Lot 422	S716-10C	0.0, 0.0, 0.0	3.9S, 1 component
S716-4F			
Lot 423	S716-24B	0.1, 0.0, 0.2	4.4S, 1 component
S716-18F	S716-10C	0.0, 0.0, 0.0	3.9S, 1 component
Lot 424	S716-24B	0.1, 0.0, 0.2	4.4S, 1 component
S717-9F	S716-10C	0.0, 0.0, 0.0	3.9S, 1 component
	D716-19C	0.0, 0.0, 0.2	4.1S, 1 component
Lot 426			
S718-20F	D717-7C	0.4, 0.0, 0.0	4.0S, 1 component
Lot 438			
S717-16F	S717-13C	0.0, 0.5, 0.3	4.7S, 1 component

B. Meningococcal Polysaccharide Vaccine, Type A.

1. Growth and Isolation Studies.

a. Growth studies.

On June 22, 1971 two lyophilized cultures from a fresh isolate of Neisseria meningitidis type A were obtained from Dr. Lowenthal, WRAIR. The culture had been evaluated under production conditions at WRAIR and shown to produce type A polysaccharide free of any type C.

A total of 6 batches (100-liters) was made and delivered for product recovery, as summarized in Table I.

The fermentation process employed was the same as that used for the meningococcal type C polysaccharide with the exception of cycle time. The fermentation cycle was shortened to 12 hours, the point of maximum growth.

Figure 1 illustrates the fermentation data of a typical batch.

b. Isolation Studies.

(1) Process Selection.

The method adopted was a modification of that published by Sanford Berman et al. of WRAIR [Infection and Immunity 2, 640-647 (1970)]. The details of the method were developed to produce a product which would meet all WRAIR chemical and biological specifications.

(2) Process.

Step #1. Collection of Wet Cells.

Each 100-liter batch of Cetavlon-treated broth was immediately centrifuged in a turbine-driven Sharples centrifuge using a No. 1-H standard clarifier bowl and revolving at 50,000 RPM and a flow rate of 30 liters/hr. The broth was followed with a wash of 1 liter of pyrogen-free water. The clear filtrate was discarded and the insoluble cake was scraped from the bowl, weighed and frozen.

Step #2. Washing.

Frozen precipitate from each batch was suspended in 600 ml of pyrogen-free distilled water and warmed to 5°C. The suspension was stirred with a Sorvall Omni-Mixer at a speed setting of 2 for 20 minutes. The mixture was centrifuged in 500 ml polyethylene cups in a Lourdes Model LRS centrifuge with a 3RA head at 6,000 RPM for 20 minutes. The wash was discarded.

Step #3. Extraction with $CaCl_2$-Solution.

Washed cells were stirred in 600 ml of 1.0M $CaCl_2 \cdot H_2O$ solution with an Omni-Mixer at a speed setting of 6 for 30 minutes. The mixture was centrifuged in the Lourdes at 6,000 RPM for 30 minutes. The clear supernate was decanted and saved. The cake was extracted two more times by the same method except that the Omni-Mixer was operated at a speed setting of 2 for 15 minutes and the centrifuge times were 20 minutes and 20 minutes, respectively. All three extracts were pooled. The residual cake was discarded.

Step #4. Ethanol Precipitation - 25%

To the combined $CaCl_2$ extracts were added 600 ml of denatured absolute ethanol (2BA). The solution was allowed to stand overnight. The 25% ethanolic solution was centrifuged in the Lourdes as before and the precipitate was discarded.

Step #5. Ethanol Precipitation - 83.5%.

The clear solution from the above was adjusted to 83.5% ethanol by the additon of 8.4 liters of denatured absolute ethanol. The solution was allowed to stand at 5°C for 3 hours. The precipitate was collected by vacuum filtration using a 10" Buchner funnel set with two sheets of Reeve-Angel #230 paper. The cake was washed with about 200 ml of ethanol, then with about 200 ml of acetone and was dried under vacuum in a desiccator over $CaCl_2$. The dried product was weighed.

Step #6. Chloroform Emulsification (Sevag).

The product from above was dissolved in 400 ml of pyrogen-free, distilled water and centrifuged in an International clinical centrifuge. The small amount of dark precipitate was discarded. A 400 ml portion of chloroform was added and the mixture was stirred with an Omni-Mixer at a speed setting of 1.5 for 30 minutes. The container was immersed in an ice bath during mixing. The mixture was then centrifuged in the Lourdes at 6,000 RPM for 20 minutes. The clear water layer was siphoned off and treated 3X more with 400 ml portions of chloroform. The clear chloroform layers were discarded and the emulsions at the interfaces were saved.

Step #7. Second Ethanol Precipitation.

The final water layer, 400 ml, was diluted with 2-liters of absolute ethanol to 83.5% concentration. The suspension

Step #7 (Cont.)

was allowed to stand overnight at 5°C, then the product was collected by centrifugation in the Lourdes centrifuge as before.

Step #8. Second Extraction with $CaCl_2$ Solution.

The wet precipitate was extracted with portions of 0.02M $CaCl_2$ solution until a total of 300 ml of extract was obtained. Between extractions the mixture was separated by centrifugation in an International clinical centrifuge. The final dark colored precipitate was discarded.

Step #9. Ultracentrifugation.

The extract was clarified by centrifugation in a Beckman Model L centrifuge with a number 30 head at 30,000 RPM for 2 hours at 5°C. The clear solution, 270 ml, was diluted with 250 ml absolute ethanol to 48% concentration. The solution was again clarified by ultracentrifugation as above. The second pellet was discarded. To the clear solution were added 5 ml saturated pH 7.0 sodium acetate plus 1250 ml of absolute ethanol. The solution was allowed to stand at 5°C overnight and the precipitate was centrifuged off. The precipitate was washed and dried with ethanol and then with acetone. The product was dried in vacuum.

The dried product was reprecipitated by dissolving in 125 ml of pyrogen-free water containing 2% 1M $CaCl_2$. To the clear solution were added 136 ml of ethanol to a concentration of 52.2% to produce opalescence. The opalescent solution was centrifuged 2 hours at 78,000 G. The clear solution was treated with 2.5 ml saturated sodium acetate (pH 7.0) plus 490 ml of ethanol to an ethanol concentration of 83.5%. The precipitate was allowed to settle overnight at 5°C. The insoluble product was centrifuged off, washed with absolute ethanol and acetone and dried in vacuum.

Step #10. Final Product.

Samples of each final product were submitted for the required assays. The final product from Batch 2A failed the pyrogen test and the last alcohol precipitation step was repeated to yield acceptable material. The final product from the other batches passed all of the required analyses. The reworked product from Batch 2A and the final product from Batch 3A were combined and blended by rotating the container on a roller mill for four hours. Batches 5A and 6A were combined at Step #2, processed using twice the quantities of materials and delivered as one lot.

(3) Delivery Lots for Formulation.

The sample numbers assigned to the deliveries for formulation were as follows:

Delivery No.	Sample No.	Batches	L-Number
1	S718-25P	2A & 3A	675,786-00B01
2	S719-15P	5A & 6A	675,786-00B02

Table II summarizes the data on each batch. A total of 8.9g was delivered for vaccine formulation in two deliveries representing an average yield of 2.5g per 100 liters of broth. Table III gives the chemical analyses for each of the two deliveries.

(4) Analyses.

Assay	Method	Specification
Phosphorus	Chen and Toribara	min. 8% (anhydrous basis)
Protein	Lowry	max. 1%
Nucleic Acid	UV spectroscopy	max. 1%
Pyrogens	3 rabbits 2.5 µg I.V. each	No more than 0.5°C average rise, no more than 1.0°C rise in any single rabbit
Molecular Weight	(a) Sephadex G-200 chromatography	Bulk of phosphorus in void volume
	*(b) Sepharose 4B chromatography	None

* Procedure described in section on Meningococcal Type C Polysaccharide.

(5) Stability.

Since meningococcal type A polysaccharide has been shown to break down to low molecular weight products, reference samples of this product were analyzed by the Sepharose 4B chromatography. Some reduction of molecular weight was observed in samples of final product which were stored at room temperature for approximately 2 months. All supplies of final product and packaged vaccine are now stored at -20°C. Samples of packaged vaccine were obtained from West Point for comparison by Sepharose 4B chromatography. The polysaccharide was separated from the salt diluent by dialysis before chromatography. All of these samples exhibit peaks corresponding to molecular weights of greater than 200,000, thus, all of the samples should be excluded from Sephadex G-200. Samples of final product and

vaccine prepared by E. R. Squibb & Sons, Inc. have been chromatographed and give very similar results on final vaccine. The molecular weight of vaccine, however, is only half that of final product.

Sample No.	Description	Date of Preparation	Date of Chromatography	Molecular Weight
S717-29B	Product from Batch 2A	7/29	8/3	4.3×10^5
S717-21C	Product from Batch 3A	7/21	8/4	5.0×10^5
S718-25P	Delivery #1 reference sample	9/10	10/5	2.6×10^5
Lot #439A	Vaccine from Delivery #1	---	9/29	2.3×10^5
S718-26D	Product from Batches 5A & 6A	8/26	9/15	4.5×10^5
S719-15P	Delivery #2 reference sample	8/26	10/8	3.5×10^5
Lot #440A	Vaccine from Delivery #2	---	10/9	3.5×10^5
Squibb #8	Final product	---	9/17	5.2×10^5
Squibb #8	Vaccine	4/70	10/13	2.2×10^5

A polysaccharide sample stored at $-20^{\circ}C$ for 3 months was observed to drop from a molecular weight of 5.2×10^5 to 3.4×10^5. Thus, it appears that $-20^{\circ}C$ does not guarantee retention of the high molecular weight of freshly isolated polysaccharide.

2. Formulation and Testing of Final Container Vaccine.

a. Vaccine formulation.

The preparation of approximately five gms. (101,500 x 50 mcg doses, 0.5 ml each) of lyophilized Neisseria meningitidis, type A polysaccharide vaccine has been completed. The vaccine has been delivered to Dr. M. Artenstein, WRAIR, along with release protocols which describe the preparation and control testing of each of the vaccine lots. The five gms. were distributed in two separate lots (50 dose containers) and each passed all contract specifications as well as control safety testing.

The control testing procedures followed for release of lot 439/C-A453 are presented in Indicies 1-9. Similar procedures were utilized in the preparation and testing of lot 440/C-A455.

(1) Rehydration and filtration of final pool.

All glassware, metal and rubber tubing used were prepared so as to eliminate any contribution of detectable pyrogens refer to Index 3). All work subsequent to the weighing step of the desiccated polysaccharide was performed under a recirculating, laminar flow hood (Air Control, Microvoid model IV BCO. All sample and sterile rubber hose connections were flamed properly with a bunsen burner. Neisseria meningitidis, type A (strain A-1a polysaccharide powder) was weighed in a glass beaker on a Mettler semi-microanalytical balance (model B6). Subsequently, the powder was added to saline solution (refer to Index 4); the same solution was used to rinse the beaker and was added to the pool. The polysaccharide solution was placed on a rotary shaker (Thomas Rotating Apparatus No. 3263, setting at 3.5) for 30 minutes at 20-25°C. A Millipore 142 mm filter holder containing a sterile 0.22 micron porosity Millipore filter (GSWP) and a Millipore prefilter (AP2012450) were primed with the saline diluent (500 to 600 ml) described above (fluid and container removed). Subsequently, the polysaccharide solution was passed through the filter under filtered air pressure, into a new container. After the filtration step, the filter pad was examined for possible breaks. The bulk, filtered solution was sterility tested.

(2) Filling of final pool.

The sterile filtered polysaccharide solution was delivered to the MSD Biological Production Laboratories for filling into final containers (Index 9). The dispensing (2.0 ml/vial) was conducted under a laminar flow hood, using aseptic techniques, into glass vials (#50276); rubber stoppers (#52531) suitable for lyophilization were inserted half way. A member of the Virus & Cell Biology staff always was present for the entire operation. An appropriate number of vials from the beginning, middle and end of the filling operation were coded and removed after the lyophilization step. The stoppered vials were placed in an electric freezer, -50 to -70°C overnight.

(3) Lyophilization of final pool.

A separate thermocouple was attached to each of four vials which were positioned in appropriate areas of the Hull lyophilization cabinet (Model 651-3PF-25F) during the operation. The shelves of the unit were cooled to -40°C, after which the trays of vials were loaded into the unit. Evacuation of the chamber was performed until the pressure was down to 175 microns, at which time the lyophilization cycle was begun; the vacuum eventually reaches approximately 39 microns of pressure. The shelf temperature was held at -40°C for 16 hours; the temperature was increased to 0°C over a 3-hr. period. The shelf temperature was held at 0°C until the material temperature

increased to -5°C; the shelf temperature was increased to +10°C and held. The material temperature was at +10°C on all thermocouples for a minimum of 6 hours before unloading The total lyophilization cycle was 45-48 hours. When the run was completed, the vacuum valves were closed and argon was admitted to a pressure reading of 3". The vial stoppers were depressed internally and sufficient argon was admitted to equilibrate atmospheric pressure.

The rubber-stoppered vials were capped with aluminum seals (flip-off red plastic cente·) and stored at -20°C in sealed cartons until they were labelled.

(4) Assays on final container vaccine.

The assays performed on final container type A polysaccharide vaccines have been described in Indicies 1-9.

Results of final container assays have been listed in Tables IV and V.

TABLE I. MENINGOCOCCAL TYPE A FERMENTOR PRODUCTION

Batch No.	Date Delivered	Wet Cell Paste (grams)
F-1	6/30/71	300
F-2	7/1/71	295
F-3	7/9/71	291
F-4	not delivered	
F-5	8/19/71	256
F-6	8/20/71	235

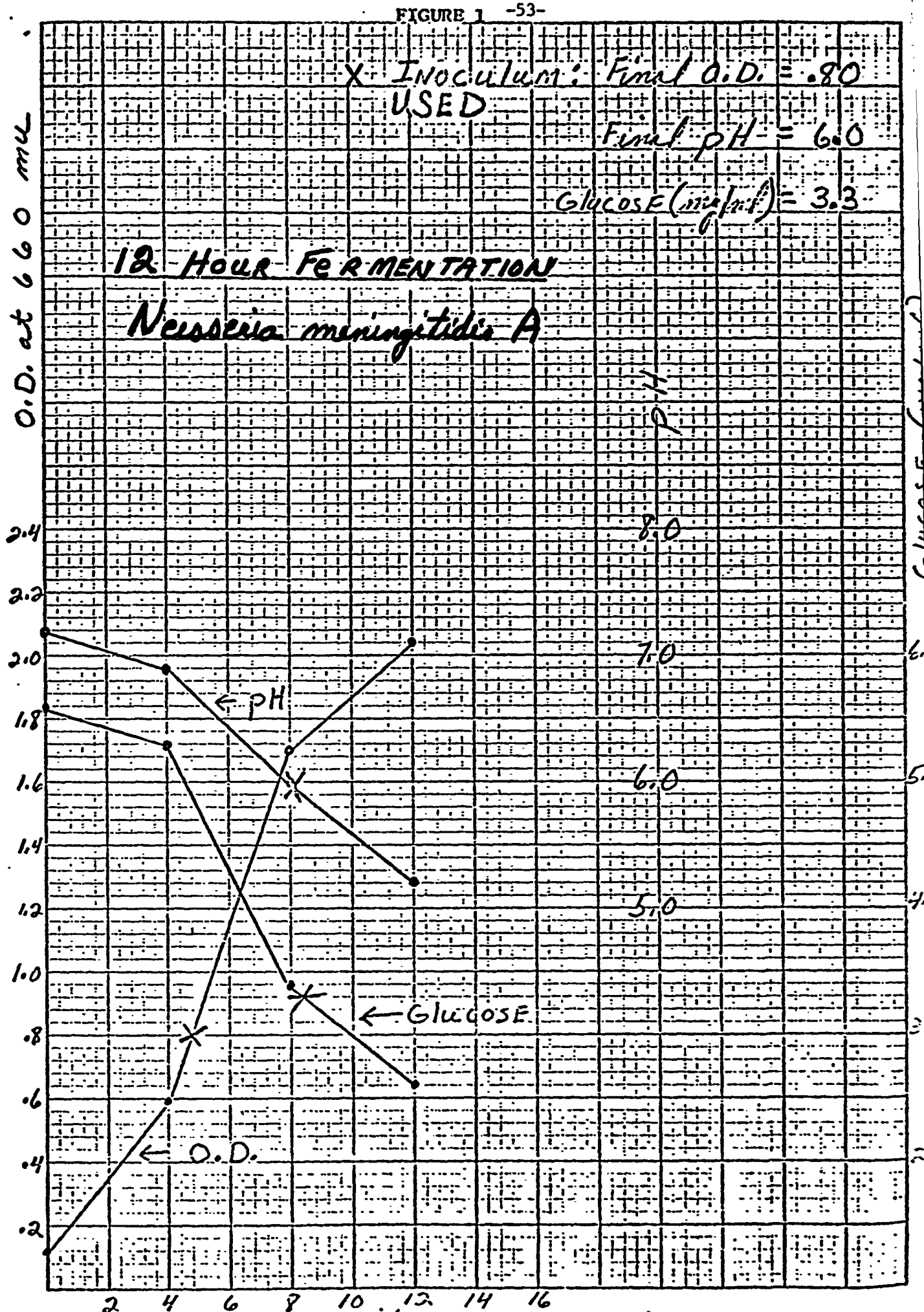
X Inoculum: Final O.D. = .80
Final pH = 6.0
Glucose (mg/ml) = 3.3
12 Hour Fermentation
Neisseria meningitidis A
O.D. at 660 mμ
pH
Glucose (mg/ml)
8.0
7.0
6.0
5.0
← pH
← Glucose
← O.D.
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
2
4
6
8
10
12
14
16
6.
5.
4.
3.
2.
KEUFFEL & ESSER CO. MADE IN U.S.A. 46 0360 5 X 5 TO ½ INCH 7 X 10 INCHES

TABLE II. BATCHES PRODUCED

Batch No.	Delivery Date	Wet Cell Weight	Yield				Phosphorus	Protein	Nucleic Acid	Sephadex* G-200	Sample No.
			Step 5	Final	Rework						
2A	7/1	295 g.	9.0 g.	2.5 g.	1.8 g.	0.0, 0.0, 0.0	8.6%	0.75%	0.3%	78%	S717-29B
3A	7/9	291	5.0	1.7	--	0.3, 0.2, 0.3	8.4	0.95	0.2	85	S717-21C
5A	8/19	256	19.2	6.4	--	0.2, 0.0, 0.2	8.4	0.5	0.5	94	S718-26D
6A	8/20	235									

* Calculated as percentage of product recovered in the void volume as determined by phosphorus determination.

TABLE III. CHEMICAL ANALYSES OF DELIVERIES

Test \ Sample	DELIVERY #1 L-675,786-00801 (S718-25P)	DELIVERY #2 L-685,786-00302 (S719-15P)
Phosphorus, % (1)	8.39	8.35
Protein, %	0.78	0.50
Nucleic Acid, %	0.2	0.5
Moisture by TGA, % (2)	7.4	7.3
Gel Filtration MW, % (3)	70.6	79.2
Sialic Acid, %	0.18	0.19
Total Acetyl, % (4)	13.6	20.1
O-Acetyl, μmol/mg (5)	2.1	2.2
Spectrographic Analysis:		
Calcium	major	major
Phosphorus	minor	minor
Iron	trace	trace
Copper	trace	trace
Sodium	trace	trace
Silicon	trace	trace
Magnesium	trace	trace

(1) On anhydrous sample

(2) Thermogravimetric analysis

(3) Number in Table is phosphorus found in void volume x 100/phosphorus charged to column

(4) By chromic acid oxidation

(5) By Ferric hydroxamate method using glucose pentaacetate as standard

MERCK SHARP & DOHME Index 1 -56- BIOLOGICAL WORK ORDE
MERCK SHARP & DOHME RESEARCH LABORATORIES
Division of Merck & Co., Inc.

OB 284

TO: BIOLOGICAL PRODUCTION, MS&D		DATE OF REQUEST: 9/23/71
FROM: Dr. Vella Virus & Cell Biology Research	DEPT. No. 874	ACCT'G CHARGES: (SPECIFY ACCT. NO. OR PROJECT NO.) 88-807-126

DESCRIPTION OF WORK REQUESTED:

CONTROL

Please perform the following animal control tests as indicated below on Meningococcal polysaccharide type A, final container: Lot 439, C-A453, L-675, 786, 00B01.

A. Pyrogen test in rabbits (2 kg weight)

1. 3 rabbits each to receive 1.0 ml (2.5 mcg, intravenously) per 2 kg. of meningococcal polysaccharide type A, to be performed on each of 2 samples.

2. 3 rabbits to receive 1.0 ml of CM 66(distilled water) placebo intravenously.

3. Record rectal temperatures of all rabbits just prior to injection and 1, 2 and 3 hours post injection.

B. Safety test in mice (14-16 gm weight)

1. 20 mice to receive 0.5 ml (100 mcg, intraperitoneally) of meningococcal polysaccharide type A, each of two samples to be tested in 10 mice (20 total

2. 20 mice to receive 0.5 ml of CM 66 (distilled water) placebo intraperitonea

3. Observe and weigh all animals daily for 7 days; record weights and submit on assay report.

C. Safety test in guinea pigs (350 gm weight)

1. 4 guinea pigs to receive 1.0 ml (500 mcg, intraperitoneally) of meningococcal polysaccharide type A, each of two samples to be tested in 2 guinea pigs (4 total).

2. 3 guinea pigs to receive 1.0 ml of 4.6% saline placebo intraperitoneally.

3. Observe, weigh and check rectal temperatures for all animals daily for 7 days. Record temperatures and weights and submit on assay report.

D. Please submit results to Dr. Vella in the form of written assay reports. A Certificate of Analysis is required also.

IF ESTIMATED COST IS LESS THAN $_____ THIS REQUEST IS VALID WITHOUT FURTHER APPROVAL.	ORIGINATED BY: Donald E. Hirsfield
	ACCEPTED BY BIOLOGICAL PRODUCTION:
ESTIMATED COST:	ESTIMATE APPROVED BY:

BIOLOGICAL WORK ORDER NO. _____ DEPT. NO. _____ RESULTS TO CONTROL

ROUTING:

1. ORIGINATOR WILL FORWARD ALL COPIES TO DIRECTOR, BIOLOGICAL PRODUCTION.
2. DIRECTOR, BIOLOGICAL PRODUCTION WILL SUPPLY ESTIMATE AND ACCEPTANCE.
3. IF ESTIMATE IS LESS THAN THE STIPULATED AUTHORIZATION, DISTRIBUTE COPIES AS FOLLOWS:
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   1 COPY TO PRODUCTION CONTROL
   3 COPIES TO BE RETAINED BY DIRECTOR, BIOLOGICAL PRODUCTION.
4. IF ESTIMATE EXCEEDS STIPULATED AUTHORIZATION RETURN COMPLETE SET TO ORIGINATOR FOR NECESSARY APPROVALS. WHEN THESE ARE OBTAINED, SET IS RETURNED TO DIRECTOR, BIOLOGICAL PRODUCTION FOR DISTRIBUTION AS OUTLINED IN PARAGRAPH 3.

Index 2

Sedimentation Analysis

The sedimentation analysis of the polysaccharide was performed in a Spinco Model E analytical centrifuge under the following conditions:

1. polysaccharide concentration* at 0.5 mg/ml
2. solution - 0.85% NaCl
3. rpm - 48,000 ($W^2 = 2.54 \times 10^7$)
4. cell - 30 mm
5. rotor - Model E
6. temperature - 20°C
7. Time - 1.5 hours

*Note: lyophilized, final container vaccine (diluted to 500mcg/ml) was dialyzed against CM258 (pyrogen-free, 0.85% saline); 2.0 ml of diluted vaccine against one liter of CM258 for 24 hours.

Index 3

Glassware, rubber and metal tubing and filter holder preparation

A. All containers (4 liter bottles with rubber tubing and stainless steel connecting pieces) were prepared in the Merck Sharp & Dohme Biological Production area (Mr. E. Lewis, West Point, Pa.) according to a standard procedure utilized for preparing such materials to eliminate possible extraneous sources of pyrogenicity. Pyrogen-free water rinses and dry wall heating of glassware were performed while tubing and metal connections were rinsed with pyrogen-free water including a step utilizing water at 180°F and subsequently autoclaved.

B. All other glassware, such as volumetric flasks and pipettes and beakers were prepared in a similar manner in the Department of Virus and Cell Biology Research, MSDRL, West Point, Pa.

C. New Millipore filter holders (142 mm) were flushed initially with four liters of boiling, pyrogen-free, distilled water. Prior to each filtration of polysaccharide solution, the pre-filter and filter were flushed with 500 to 600 ml of pyrogen-free polysaccharide diluent (11.25% saline X CM61). After filtration, the filter units were flushed with three to four liters of pyrogen-free water (CM66) and autoclaved immediately.

PREPARATION OF PYROGEN-FREE EQUIPMENT

Glassware is washed either on the Better Built Hydromatic or Turbomatic washer and subjected to the treatment described below.

Hydromatic Washer

1. Pre-rinse - recirculated water
2. Wash cycle - recirculated detergent charged water, heated to 160°
3. Rinse - recirculated tap water from rinse tank, heated to 160°
4. Rinse - direct hot tap water, 150°
5. Rinse - pyrogen-free distilled water, 180°

Turbomatic Washer

1. Pre-rinse - recirculated water
2. Wash cycle - recirculated hot tap water charged with detergent, Aura, Temperature 160°
3. Rinse - recirculated hot tap water, Temperature 160°
4. Rinse - recirculated pyrogen-free water, Temperature 180°
5. Rinse - direct pyrogen-free rinse, Temperature 180°

Rubber tubing and machine components are subjected to the treatment described below.

Tumbler Washer

1. Tubing and components placed in barrel of tumbler and covered with hot tap water.
2. Boil tubing for full five (5) minutes. (No detergent used.)
3. Rinse - Pyrogen-free distilled water, 180° temperature for five (5) minutes while tumbling.
4. Rinse - Pyrogen-free distilled water, 180° temperature for five (5) minutes while tumbling.
5. Drain and jog to eliminate excess water in tubing.

Machines Assembly

1. Cut tubing to proper lengths.
2. Inspect stoppers, S.S. connectors and S.S. 'L's.
3. Plug air filter and insert in tubing; connect to SS. 'L'.
4. Assemble tubing, stoppers, and S.S. components.
5. Rinse all parts with pyrogen-free distilled water.
6. Wrap exterior ends of machines using gauze, paper envelopes, and Twistems.
7. Place Diack control on S.S. 'L'.

Index 3 (cont.)

Assembly of Machine and Bottle Units

1. Inspect bottles and machines.
2. Add approximately 10-25 cc pyrogen-free distilled water to bottle.
3. Insert machine in bottle.
4. Adjust 'C' clamp to hold stopper/machine firmly in bottle.
5. Cover stopper and neck of bottle with aluminum foil.

Sterilization is by autoclave at 250°F. for a minimum of 30 minutes.

8/23/71

Index 4

Polysaccharide Diluent

A sodium chloride solution of 11.25% (XCM-61) is required so that rehydration of the polysaccharide (1:12.5x dilution) to 100 mcg/ml will yield approximately 9.0 mg NaCl/ml. In order to compensate for the 11.69% value obtained for the XCM-61, 40.0 ml of pyrogen-free, distilled water were added to each 1000.0 ml of 11.69% saline solution to yield a final concentration of 11.25%.

Index 5

Microbial Sterility

A. Post-filtration, bulk pool

1. Three to 5.0 ml of the polysaccharide concentrate (1.25 mg/ml) are inoculated into each of four bottles of fluid thioglycollate medium (150 ml medium per bottle) and incubated at 32°C for 14 days. The bottles are examined two to three times prior to the final reading for evidence of microbial contamination.

2. Three to 5.0 ml of the polysaccharide concentrate (1.25 mg/ml) are inoculated into each of four bottles of trypticase soy medium (150 ml medium per bottle) and incubated at 20-25°C for 14 days. The bottles are examined two to three times prior to the final reading for evidence of microbial contamination.

B. Lyophilized, final product, lot 439, C-A453

1. Six (6), 8 and 6 vials were obtained from the beginning, middle and end of the filling operation respectively. One (1.0 ml) was removed from each of the 20 vials after reconstitution with 25.0 ml distilled water to give a polysaccharide concentration of 100.0 mcg/ml and inoculated into 20 bottles of fluid thioglycollate medium (150 ml medium per bottle), incubated and observed as indicated as in A. 1. above. Additionally 1.0 ml was removed from each container and inoculated into 20 bottles of trypticase soy medium (150 ml medium per bottle), incubated and observed as indicated in A. 2. above.

MERCK SHARP & DOHME
MERCK SHARP & DOHME RESEARCH LABORATORIES
Division of Merck & Co., Inc.

BIOLOGICAL WORK ORDER

OB 284

TO:		DATE OF REQUEST:
BIOLOGICAL PRODUCTION, MS&D		9/23/71
FROM: Dr. Vella Virus & Cell Biology Research	DEPT. No. 874	ACCT'G CHARGES: (SPECIFY ACCT. NO. OR PROJECT NO.) 60-807-126

DESCRIPTION OF WORK REQUESTED:

CONTROL

Sterility Tests

Please check the indicated number of final containers of the indicated lot of Meningococcal polysaccharide for sterility in trypticase soy and fluid thioglycollate medium according to the latest Public Health Service Regulations.

1. Lot 439, C-A453 -20 final containers (labeled 1, 2 and 3)

Add 1.0 ml of polysaccharide solution per bottle of testing medium. The fluid thioglycollate medium should be incubated at 32° for 14 days while the trypticase soy medium should be incubated at 20 - 25° C for 14 days. Please submit results to Dr. Vella in the form of written assay reports. A Certificate of Analysis is required also.

BIOLOGICAL WORK ORDER NO. 1715
REPORT RESULTS TO CONTROL OFFICE
CERTIFICATE OF ANALYSIS WILL BE PREPARED BY BIOLOGICAL CONTROL OFFICE.

IF ESTIMATED COST IS LESS THAN $_____ THIS REQUEST IS VALID WITHOUT FURTHER APPROVAL.

ORIGINATED BY: Kenneth E. Hadfield

ACCEPTED BY BIOLOGICAL PRODUCTION:

ESTIMATED COST:

ESTIMATE APPROVED BY:

ROUTING:

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4. IF ESTIMATE EXCEEDS STIPULATED AUTHORIZATION RETURN COMPLETE SET TO ORIGINATOR FOR NECESSARY APPROVALS. WHEN THESE ARE OBTAINED, SET IS RETURNED TO DIRECTOR, BIOLOGICAL PRODUCTION FOR DISTRIBUTION AS OUTLINED IN PARAGRAPH 3.

Index 6

BIOLOGICAL TESTING PROCEDURE

PHARMACEUTICAL LABORATORY

Rec'd 6/30/71 O.D.

I. SAMPLES

Final container samples of completed product shall be tested. Individual samples having a low net weight shall be pooled at the time of testing in order to attain an adequate sample weight.

II. TEST PROCEDURE

A minimum of 100mg. of dried product obtained from one or more final containers shall be used. The test shall consist of determining the weight loss of the sample(s) due to drying for 72 hours under vacuum over phosphorous pentoxide.

A. Recommended materials and equipment:

1. Numbered, low form, flat-bottom weighing dishes with well-ground glass stoppers
2. Vacuum desiccator equipped with a vacuum pump and an uncalibrated manometer
3. Balance capable of accurate readability to 0.1 mg.
4. Low humidity room equipped with hygrometer and maintained at a relative humidity of 0 - 10%. All operations are performed in this room.
5. Desiccator jar equipped with silica gel drying agent with indicator

B. Method:

1. Thoroughly cleaned weighing dishes shall be dried by hand and stored in the low humidity room for 24 hours prior to use. NOTE: All equipment is kept in the low humidity room at all times.
2. The weighing dishes shall be weighed as rapidly as possible. All manipulations of weighing dishes shall be made with tongs or while wearing gloves.

B. Method (Cont'd)

3. The vacuum in the sample bottle shall be released by allowing dry air to enter slowly through a needle inserted in the stopper.

4. The stopper shall be removed and the sample plug broken up with a spatula.

5. The sample shall be rapidly transferred to a previously weighed and marked weighing dish and covered with its lid.

6. After all transfers have been completed, the weighing dishes with their contents shall be weighed immediately.

7. The samples and the weighing dishes with the lids set perpendicular to the normal position shall be placed in the vacuum desiccator. The pressure shall be reduced as indicated by the manometer and the reduced pressure maintained by sealing the desiccator.

8. After 72 hours of drying time, dry air is allowed to continually bleed into the desiccator until the pressure inside has been equalized with the atmosphere.

9. The lids shall be immediately replaced in the normal closed position.

10. The weighing dishes containing the samples shall be removed individually from the desiccator and weighed as rapidly as possible.

11. All samples are to be tested in duplicate and run with a standard in each desiccator.

C. Calculations:

1. Record weights in Steps 2, 6, and 10.

2. Weight in Step 2 equals tare weight of dish.

3. (Weight in Step 6) minus (weight in Step 1) equals weight of sample before assay.

4. (Weight in Step 6) minus (weight in Step 10) equals weight equivalent to residual moisture of sample.

5. (Weight equivalent to moisture) divided by (weight of sample) times 100 equals the percentage of residual moisture.

III. REQUIREMENTS

A. Final container samples of completed product shall be used. Sample weight shall be 100 to 1,000 mg.

B. If only one sample is tested, the serial or subserial is unsatisfactory if the moisture content exceeds 2.0% for vaccines only.

C. If any determination is greater than 2.0%, a repeat test must be done.

D. The repeat test will consist of four determinations run in duplicate on two separate days. The lot will only be considered satisfactory if no values on repeat determinations are above the 2.0% limit and the average of all six determinations is below the 2.0% limit.

E. In the event that one of the two determinations is over 2.0%, a retest will be done.

The repeat test will consist of two determinations. The lot will only be considered satisfactory if no values on repeat determinations are above the 2.0% limit and the average of all four determinations is below the 2.0% limit.

NOTE:

'Dormovac' is always assayed on the Moisture Monitor.

1. Only the content of one vial is used.
2. The sample is weighed on the Microbalance (accuracy to 0.01 mg.).
3. Sample is tested in triplicate.
4. Calculation:

$$\frac{\text{Moisture Monitor reading in mg.}}{\text{Sample weight in mg.}} \times 100\% = \%\ \text{moisture}$$

12/22/70

Index 7

Determination of Chloride in RCM6 or RCM8

. (based on a modification of Swain, J.S., Chemistry and Industry, 1956, pp. 418-420)

I. Preparation of Standards

A. Weigh 2.103 gms of C.P. KCl into a 1-liter volumetric flask, dissolve and dilute to mark with RCM2, thus preparing a solution containing 1000 mcg/ml Cl^-. Dilute appropriately with RCM2, using volumetric pipettes and flasks, to obtain standard solutions containing 20, 22.5, 25, 27.5 and 30 mcg/ml Cl^-.

II. Preparation of Samples

A. Using volumetric pipettes and flasks, dilute 5.0 ml of samples to 1000 ml with RCM2. Since RCM6 and RCM8 each theoretically contains 5368 mcg/ml Cl^-, this will give a solution of the sample containing approximately 26.84 mcg/ml Cl^-.

III. Chemicals Used

Acetone, ACS Reagent Grade
Methanol, ACS Reagent Grade
Ferric Alum, C.P.
Nitric Acid, Conc., C.P.
Mercuric Thiocyanate (may be Fisher Certified or made from C.P. $Hg(NO_3)_2$ and C.P. KSCN)
RCM2 (glass distilled water)

IV. Preparation of Reagents - for Beckman DU Procedure

A. $Hg(SCN)_2$ - Dissolve 300 mgs of H[illegible] in 100 ml methanol. Filter if necessary.

B. Ferric Alum - Dissolve 6.0 gms Fer[illegible] Alum in 100 ml 6N nitric acid (39 ml conc. nitric acid plus 61 ml RC[illegible]). Filter thru fine sintered glass.

V. Procedure - Beckman DU

A. In a series of 6 x 7/8" test tubes, pipette 5.0 ml of RCM2 (for 0 blank), standard solutions and replicate sample solutions. To each tube add 5.0 ml of acetone. Mix. Add 1.0 ml ferric alum reagent. Mix. Add 1.0 ml $Hg(SCN)_2$ reagent. Mix, let stand 15 minutes and read λ470. Plot data and calculate concentrations in the usual way.

VI. Preparation of Reagents - for Spectronic 20 Procedure

A. $Hg(SCN)_2$ - Dilute one volume of the $Hg(SCN)_2$ solution described above with 9 volumes of acetone.

B. Ferric Alum - Dilute 2 volumes of the ferric alum reagent described above with 3 volumes of RCM2.

VII. Procedure - Spectronic 20

A. In a series of 6 x 7/8" test tubes, pipette 5.0 ml of RCM2 (for 0 blank), standard solutions and replicate sample solutions. Add 5.0 ml of ferric alum reagent, mix. Add 10.0 ml $Hg(SCN)_2$ reagent, mix. Let stand 15 min. and read at λ470. Plot data and calculate concentrations in the usual wa

VIII. Notes

A. The optical density scale of the Spectronic 20 is so constructed that readings can be made much more accurately in the 0.05-0.3 region than in the 0.3 to 1.0 region. This is not true to a significant degree with the Beckman DU. Accordingly, the procedure used with the Beckman DU has been modified for use with the Spectronic 20. The latter procedure has only 1 the sensitivity of the former but is quite usable.

B. The color developed in this procedure is quite stable; there is a loss of approximately 1% in a 45-minute period following the usual reading time.

C. This method is not affected by even large concentrations of phosphate.

IX. Calculations

A. For physiological saline (RCM6) and phosphate buffered physiological salin use the following:

1. Average reading of sample x 200 (dilution) x 27.5 = mcg Cl^- in sample, average reading of 27.5 standard.

2. Cl^- = 60.8% of the weight of the NaCl molecule.

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Meningococcal (type A) Hemagglutination Procedure

Reagents

Human 0, Rh negative red blood cells (Behringwerke diagnostics - type-a-cells 35 kit)
RCM8 - store at 4°C
Bovine serum albumin 10% (sterile) store at 4°C
Meningococcal antigen (polysaccharide) stored at -70°C; thawed at 37°C
Cooke Microtiter plates and seals
Microtiter loops (0.05 ml)

Procedure

1. Wash human 0 negative red blood cells three (3) times in RCM8.
2. Resuspend after final wash to 2%.
3. Add appropriate amount of antigen (determined by grid titration) to the cells.
4. Mix thoroughly, incubate 30 min. (or longer) in 37°C water bath, mix and invert every 5-7 minutes.
5. Wash RBC-antigen three (3) times in RCM8 and resuspend to a 0.5% suspension in RCM8.
6. Add bovine serum albumin to a final concentration of 0.5% w/v. (1 ml of 10% BSA +19 ml rbc-antigen).

   a. Defrost sera, heat at 56°C for 30 minutes.

   b. Prepared serial 2-fold dilutions (1:2 - 1:2048) in RCM8.

   c. 0.05 ml RBC-antigen suspension is added to each well.

   d. Mix gently.

   e. Incubate plates 2-4 hrs. at 4°C, 20 minutes at room temperature and then read for HA.

MERCK SHARP & DOHME
MERCK SHARP & DOHME RESEARCH LABORATORIES
Division of Merck & Co., Inc.

BIOLOGICAL WORK ORDER

OB 284

TO:		DATE OF REQUEST:
BIOLOGICAL PRODUCTION, MS&D		8/19/71
FROM: Dr. P. Vella Virus & Cell Biology Research	DEPT. No. 874	ACCT'G CHARGES: (SPECIFY ACCT. NO. OR PROJECT NO.) 88-807-126

DESCRIPTION OF WORK REQUESTED:

PRODUCTION

Please arrange for filling, lyophilization, labelling and packing into separator-type boxes the following lots of Meningococcal type A polysaccharide vaccine.

1. Lot 439, C-A453, L-675, 786-00B01
2. Lot 440, C-A455, L-675, 786-00B02

The sterile solutions of the type A polysaccharide will be at a concentration of 1.25 mg/ml. The number of vials (♦1100-1200 for each vaccine; 2.0 ml fill), vial size, procedures and requirements etc. will be the same as those used for the eight lots of type C polysaccharide prepared recently. The material will be available by September 13.

Appropriate
~~Approximate~~ copies of notebook records, with dates, procedures etc. used for the filling and lyophilization of these lots are requested.

Note: The lyophilization cycle should be cleared through appropriate Walter Reed Army Institute Personnel. The lyophilized product should be stored at -20° C.

A copy of the label requirements has been attached to this work order.

IF ESTIMATED COST IS LESS THAN $_____ THIS REQUEST IS VALID WITHOUT FURTHER APPROVAL.

ORIGINATED BY: Kenneth E. Hadfield

ACCEPTED BY BIOLOGICAL PRODUCTION:

ESTIMATED COST:

ESTIMATE APPROVED BY:

ROUTING:

1. ORIGINATOR WILL FORWARD ALL COPIES TO DIRECTOR, BIOLOGICAL PRODUCTION.
2. DIRECTOR, BIOLOGICAL PRODUCTION WILL SUPPLY ESTIMATE AND ACCEPTANCE.
3. IF ESTIMATE IS LESS THAN THE STIPULATED AUTHORIZATION, DISTRIBUTE COPIES AS FOLLOWS:
   2 COPIES TO ORIGINATOR (IF ORIGINATED IN RESEARCH ONE OF THESE WILL BE FORWARDED TO RESEARCH ADM., WEST POINT)
   1 COPY TO PRODUCTION CONTROL
   3 COPIES TO BE RETAINED BY DIRECTOR, BIOLOGICAL PRODUCTION.
4. IF ESTIMATE EXCEEDS STIPULATED AUTHORIZATION RETURN COMPLETE SET TO ORIGINATOR FOR NECESSARY APPROVALS. WHEN THESE ARE OBTAINED, SET IS RETURNED TO DIRECTOR, BIOLOGICAL PRODUCTION FOR DISTRIBUTION AS OUTLINED IN PARAGRAPH 3.

TABLE IV

Neisseria meningitidis, type A polysaccharide, final container assays

Vaccine Designation	Amount Weighed Gm.	Diluent Vol. ml	NaCl %	Pyrogen resp. Max. rise, °C/rabbit (2 vials)	Sterility TBS* # contam./# inoc.	Thio*	Animal Safety Tests** Guinea Pig 7d wt. gain gm B	E	P	Mouse 7d wt.gain gm B	E	P	Sedimentation+ Analysis	Moisture Content,% (2 vials)	Phosp. %	Identity (HA assay) Rabbit antiserum
439, C-A453 L675,786-00801,S717-25P	2.9225	2338	0.86	0.0, 0.0, 0.0 0.0, 0.0, 0.0	0/20	0/20	35.0++	30.0	28.0 (43.3)++	7.4	7.4	7.5	2.9S, 1 comp.	0.42, 0.38	8.1	Satisfactory
440, C-A455 L675,786-00802,S719-15P	3.1133	2491	0.93	0.0, 0.2, 0.1 0.0, 0.2, 0.0	0/20	0/20	57.5	25.0	48.3	7.06	8.0	7.7	3.0S, 1 comp.	0.1, 0.15	7.6	Satisfactory

* trypticase soy broth and fluid thioglycollate medium incubated at 20-25°C and 32°C, respectively, for 14 days

** samples taken from the beginning and end of the final container dispensing operation, each were injected into 2 guinea pigs (4 total and 10 mice (20 total); the placebo was injected into a total of 3 guinea pigs and 20 mice

\+ Spinco model E ultracentrifuge

++ Repeat assay value

TABLE V

Neisseria meningitidis, type A polysaccharide, initial batch pyrogen & sedimentation analysis assays

Vaccine Designation	Component batches, designation	Pyrogen responses, max. rise, °C/rabbit	Sedimentation analysis
Lot 439	S717-29B	0.0, 0.0, 0.0	2.9S, 1 component
S717-25P	S717-21C	0.3, 0.2, 0.3	3.0S, 1 component
Lot 440 S719-15P	S718-26D	0.2, 0.0, 0.2	3.3S, 1 component

C. Diluent for Resuspension of Meningococcal Polysaccharide Vaccines, Types A and C.

Four lots of pyrogen-free distilled water sufficient to rehydrate 548,000 doses of meningococcal polysaccharide vaccines, types A and C, were prepared and delivered to Dr. Artenstein, WRAIR. These were without preservative. They were identified as follows:

1) lot 440064/20438/C-A487 (sufficient to rehydrate 105,550 x 0.5 ml doses).

2) lot 437717/18327/C-A484 (sufficient to rehydrate 141,200 x 0.5 ml doses).

3) lot 437718/18328/C-A458 (sufficient to rehydrate 171,750 x 0.5 ml doses).

4) lot 440065/20440/C-A590 (sufficient to rehydrate 130,000 x 0.5 ml doses).

Certificates of Analysis presenting the control test results for each of the lots of diluent were provided to Dr. Artenstein along with the supplies.