[Chem. Pharm. Bull. 33(1) 333—339 (1985)]

Structure of the Polysaccharide Moiety of the *Klebsiella* O3 Lipopolysaccharide Isolated from Culture Supernatant of Decapsulated Mutant (*Klebsiella* O3:K1$^-$)

TAKAAKI HASEGAWA,*[,a] MICHIO OHTA,[a] IZUMI NAKASHIMA,[b] NOBUO KATO,[a] KIYOSHI MORIKAWA,[c] TADASHI HARADA,[c] and TAKASHI OKUYAMA[c]

Department of Bacteriology[a] and Department of Immunology,[b] Nagoya University School of Medicine, Showa-ku, Nagoya, 466, Japan, and Tokyo Institute, Seikagaku Kogyo Co., Ltd.,[c] Chuo-ku, Tokyo 103, Japan

(Received June 15, 1984)

In our earlier studies, the *Klebsiella* O3 lipopolysaccharide (LPS) isolated from culture supernatant of *Klebsiella pneumoniae* strain Kasuya (O3:K1) or its decapsulated mutant strain LEN-1 (O3:K1$^-$) was shown to exhibit a much stronger adjuvant effect than other known adjuvants, including LPS of *Escherichia coli* and *Salmonella*. The O3 LPS isolated from culture supernatant of *Klebsiella* strain LEN-1 was degraded into 59—66% neutral sugars and 25—27% lipids by hydrolysis with 1% acetic acid at 100 °C for 1 h. The polysaccharide moiety (OPS) obtained by the hydrolysis was homogeneous on gel filtration. It contained 92.4% mannose, 0.15% 2-keto-3-deoxyoctonate and less than 0.1% P. The molecular weight values of OPS determined by gel filtration analysis and by the Somogyi–Nelson method were 14000 and 16200, respectively. The specific rotation of OPS was +88° and its infrared spectrum showed no β-configuration of mannopyranose. Methylation analysis indicated that OPS contained (1→2)- and (1→3)-linkages in a ratio of 3:2. Smith degradation and partial acid hydrolysis of OPS liberated mannosyl-(1→3)-mannose. We postulate that OPS consists of a mannan which has α-mannosyl-(1→3)-α-mannosyl-(1→2)-α-mannosyl-(1→2)-α-mannosyl-(1→2)-α-mannose units joined through α-mannosyl-(1→3)-linkages.

Keywords——*Klebsiella* O3 lipopolysaccharide; *Klebsiella pneumoniae*; polysaccharide moiety; specific rotation; methylation analysis; Smith degradation

Introduction

Previously we have demonstrated that the polysaccharide-rich fraction isolated from culture supernatant of *Klebsiella pneumoniae* type 1 strain Kasuya (O3:K1) exhibits a strong adjuvant effect on antibody responses in mice to various antigens,[1—4] and that the effect is much stronger than that of other known adjuvants, including Freund's complete adjuvant and lipopolysaccharides (LPS) of *Escherichia coli* O111 and O55.[1,5,6] The polysaccharide-rich fraction isolated from culture supernatant of *K. pneumoniae* has been fractionated to the acidic polysaccharide fraction (the type-specific capsular antigen) and the neutral polysaccharide.[7] Further studies have revealed that the substance exhibiting the strong adjuvant effect is the neutral polysaccharide fraction,[7] that the decapsulated mutant strain LEN-1 (O3:K1$^-$) obtained from strain Kasuya produces the neutral polysaccharide in an amount nearly equal to that produced by the parent strain,[8] and that the neutral polysaccharide is serologically identical to the O3 antigen (O3 LPS) of *Klebsiella*.[9] In our earlier studies dealing with chemical structure of the O3 LPS, LPS isolated from culture supernatant of strain Kasuya was analyzed.[10] In the present study we have tried to characterize chemically the polysaccharide moiety of the *Klebsiella* O3 LPS isolated from culture supernatant of the

decapsulated mutant strain LEN-1 (O3 : K1$^-$).

Experimental

Isolation of the *Klebsiella* O3 LPS from Culture Supernatant——The decapsulated mutant strain LEN-1 was grown at 37 °C for 2 d in 20 l of synthetic liquid medium containing 20 μg/ml leucine with aeration according to the procedures described by Batshon *et al.*[11] At the end of culture, the culture broth was concentrated to about 2 l in a rotary evaporator at 37 °C. After removal of the cells by centrifugation, ethanol was added to the supernatant to a concentration of 67%, and the precipitated crude LPS was collected by centrifugation, washed successively with ethanol and ether, and dried *in vacuo*. The yield was about 250 mg per 20 l of the culture broth. The product was purified by treatment 3 times with a half volume of chloroform–1-butanol (5 : 1) mixture for deproteinization.[3] After removal of the precipitate thus formed, the supernatant was dialyzed against tap water and concentrated to a small volume, and LPS was precipitated by addition of ethanol. Dissolution in distilled water and precipitation with ethanol were repeated 3 times. Then, a white powder was obtained by lyophilization. The yield was 200 mg per 20 l of medium.

Separation of the Polysaccharide Moiety (OPS) and Lipid Moiety from the *Klebsiella* O3 LPS——The O3 LPS (100 mg) was dissolved at a concentration of 1% with 1% acetic acid and hydrolyzed at 100 °C for 1 h. After removal of precipitated lipid by centrifugation, the water-soluble product was washed with chloroform, dialyzed against distilled water and lyophilized to give the polysaccharide moiety (OPS) from the O3 LPS (yield, 56 mg). The sediment obtained after centrifugation of the hydrolyzate was extracted 3 times with chloroform. The chloroform-soluble products from the sediment were collected and the solution was evaporated to give the lipid moiety (22 mg) from the O3 LPS.

Gel Filtration——Sephadex G-150 (Pharmacia Fine Chemicals, Uppsala, Sweden) was washed with distilled water, then with 1 M NaCl. OPS dissolved in saline was applied to the column (2.6 × 100 cm) and eluted with the same solution at a flow rate of 25 ml per 1 h. Fractions of 3 ml were collected, and an aliquot of each fraction was assayed for carbohydrate content by the phenol–sulfuric acid method.[12] We used blue dextran 2000, dextran T-70, T-40 and T-10 (Pharmacia Fine Chemicals, Uppsala, Sweden) as markers for molecular weight determination.

Analytical Methods——OPS was dissolved in distilled water and the ultraviolet absorption spectrum was recorded with a Hitachi automatic spectrophotometer, model 333. The specific optical rotation of OPS was determined with a Jasco DIP-180 automatic polarimeter. Infrared spectra were obtained with a Hitachi infrared spectrophotometer, model EPL-3G, using the potassium bromide pellet method. The contents of carbon, hydrogen and nitrogen in OPS were determined by elemental analysis after drying the sample *in vacuo* over P_2O_5 for 24 h. Phosphorus was estimated by the method of Chen *et al.* as described by Nowotny.[13] Neutral sugar content was quantitiated by the phenol–sulfuric acid method using mannose as the standard. 2-Keto-3-deoxyoctonate (KDO) was estimated by the method of Waravdekar and Saslaw.[14] Hexosamine was determined by the method of Elson and Morgan[15] after hydrolysis of the sample in 6 N HCl at 100 °C for 5 h, using D-glucosamine hydrochloride as the standard. Protein was estimated by the method of Lowry[16] using recrystallized bovine serum albumin as the standard.

Determination of Monosaccharide Composition——OPS (10 mg) was hydrolyzed with 2 ml of 1 N H_2SO_4 at 100 °C for 10 h. After addition of $BaCO_3$, the precipitate formed was removed by centrifugation. The supernatant was examined by thin-layer chromatography (TLC). TLC on Kieselgel (0.25 mm thickness, without fluorescent indicator, Merck, Darmstadt, Germany) was carried out with the following solvent systems: A, 1-butanol–acetic acid–water (4 : 1 : 5); B, ethyl acetate–methanol–acetic acid–water (60 : 15 : 15 : 10). Sugars were detected with 25% H_2SO_4 and alkaline silver nitrate.[17]

Methylation Analysis——OPS (100 mg) was dissolved in 10 ml of dimethylsulfoxide and methylated with a solution of methylsulfinyl carbanion in dimethylsulfoxide and methyl iodide by a modification of the method described by Hakomori *et al.*[18] The resultant solution was dialyzed against tap water then extracted twice with chloroform, and the organic phase was evaporated. The residue was extracted with ether. The ether solution was dried over anhydrous sodium sulfate and then evaporated to a syrup under reduced pressure. The methylation procedure was repeated three times. Permethylated OPS (10 mg) was dissolved in 90% formic acid (2 ml), and the mixture was heated at 100 °C for 2 h and then heated with 1 N H_2SO_4 (2 ml) at 100 °C for 4 h in a sealed tube. Removal of the acid was accomplished by addition of $BaCO_3$, and the supernatant solution was evaporated to dryness under reduced pressure. The methylated monosaccharides thus obtained were dissolved in 2 ml of water and reduced with $NaBH_4$ overnight at room temperature. Dowex 50 × 8 (H-form) resin was added to the mixed solution to decompose the excess reagent. The resin was filtered off, and the filtrate was co-evaporated repeatedly with methanol to remove boric acid. The syrupy product was dried, then pyridine and acetic acid anhydride (1 ml) were added and the mixed solution was heated at 100 °C for 30 min. The partially methylated alditol acetates thus obtained were analyzed by gas chromatography (GC). The gas chromatographic analysis was carried out with a Shimadzu GC-4A on OV-17 (3% on 100—120 mesh Gaschrom Q, 2 m) at 175 or 210 °C. Analysis of the alditol acetates by GC-mass spectrometry (MS)

was carried out using a Shimadzu LKB 9000 mass spectrometer.

Smith Degradation of OPS——Smith degradation was carried out by a modification of the method described by Smith and Van Cleve.[19] OPS (50 mg) was treated at 5 °C in the dark with 50 ml of 0.04 M $NaIO_4$ under magnetic stirring. After 10 h, a few drops of ethylene glycol were added to the reaction mixture to destroy the excess periodate, and the mixture was dialyzed against tap water. The non-dialyzed substance was reduced with $NaBH_4$ (200 mg) for 24 h at room temperature. The excess $NaBH_4$ was destroyed by acidification with acetic acid under external cooling in ice water, and the reaction mixture was dialyzed and lyophilized. The yield of the product (polysaccharide polyalcohol) was 38 mg.

Partial Acid Hydrolysis: The polysaccharide polyalcohol was hydrolyzed with 0.25 N H_2SO_4 at room temperature for 72 h. The acid was removed by precipitation with $BaCO_3$, and the supernatant solution was evaporated to dryness under reduced pressure. The hydrolyzate obtained was methylated by the method described above. The derivatives obtained were hydrolyzed to methylated monosaccharides, which were converted into alditol acetates as described above. The resulting mixture of *O*-methyl-*O*-acetylmannitols was subjected to GC and the peaks obtained were further analyzed by MS.

Complete Acid Hydrolysis: The polysaccharide polyalcohol was hydrolyzed with 1 N H_2SO_4 at 100 °C for 10 h. The hydrolyzate was neutralized with $BaCO_3$ and centrifuged, and the supernatant was concentrated to a small volume. The concentrate was examined by TLC.

Results

Chemical Properties of the O3 LPS Isolated from Culture Supernatant of *Klebsiella* O3:K1$^-$

The O3 LPS was degraded by hydrolysis with 1% acetic acid at 100 °C for 1 h into 59—66% polysaccharide and 25—27% lipid (Table I). The preparation of the O3 LPS treated 3 times with chloroform–1-butanol (5 : 1) for deproteinization still contained a small amount of protein. We have shown elsewhere that a preparation of O3 LPS free from contaminating protein can be obtained by treatment with pronase followed by treatment with chloroform–1-

Table I. Chemical Composition (%) of the *Klebsiella* O3 LPS

Carbohydrates			Lipid	Protein
Mannose equivalent	Hexosamine	KDO		
59—66	6.0	2.3—2.4	25—27	2.1—2.6

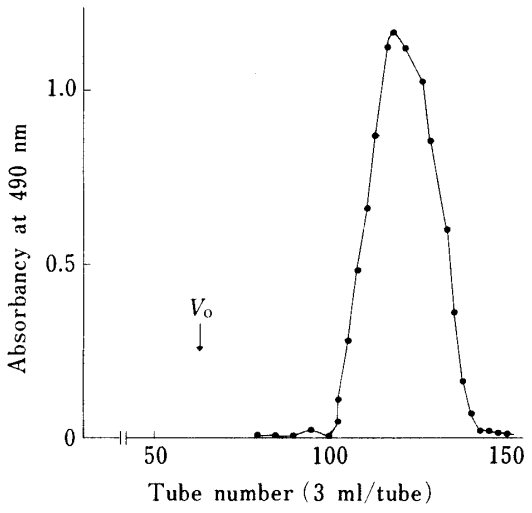

Fig. 1. Gel Filtration of OPS on a Sephadex G-150 Column

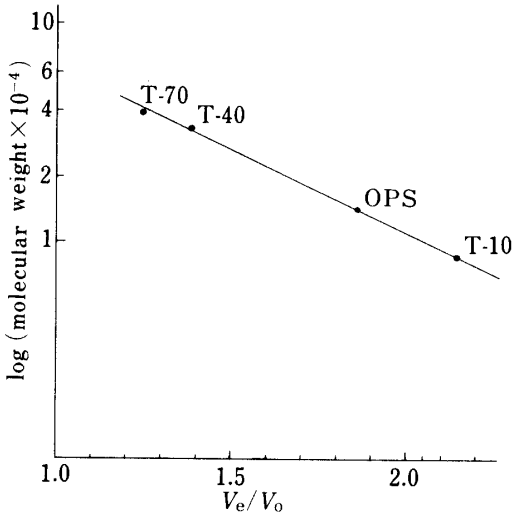

Fig. 2. Estimation of the Molecular Weight of the Substance Corresponding to the Peak Shown in Fig. 1

V_0, void volume; V_e, elution volume.

Table II. Chemical Composition (%) of OPS

C	H	N	P	Mannose	Glucose	Hexosamine	KDO	Protein
38.90	5.70	0.10	<0.1	92.4	trace	0.87	0.15	0.14

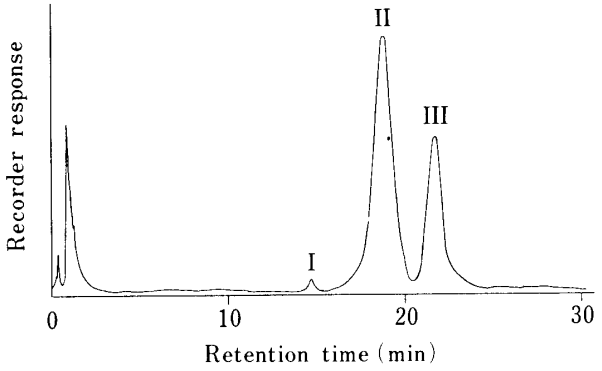

Fig. 3. GC Analysis of Partially Methylated Alditol Acetates Prepared from Permethylated OPS

Peaks I, II and III represent the derivatives of 2,3,4,6-tetra-*O*-methylmannose, 3,4,6-tri-*O*-methylmannose and 2,4,6-tri-*O*-methylmannose, respectively.

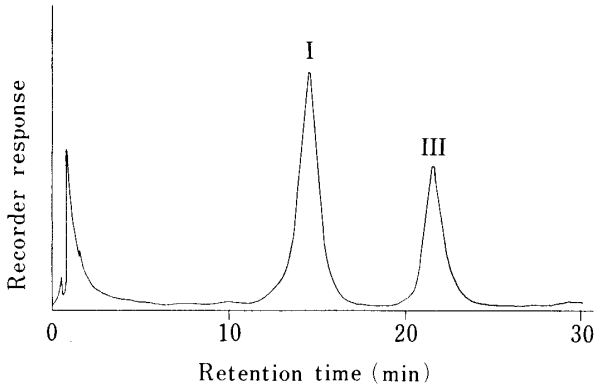

Fig. 4. GC Analysis of Partially Methylated Alditol Acetates Obtained by Smith Degradation

Peaks I and III represent the derivatives of 2,3,4,6-tetra-*O*-methylmannose and 2,4,6-tri-*O*-methylmannose, respectively.

butanol.[10]

Chromatography of OPS on a Sephadex G-150 Column

When OPS was applied to a Sephadex G-150 column, it gave a single peak (Fig. 1), suggesting that OPS was homogeneous. The approximate molecular weight of OPS estimated by gel-filtration on a Sephadex G-150 column was 14000 (Fig. 2).

Physico-Chemical Properties of OPS

OPS was readily soluble in water. The approximate molecular weight of OPS was also estimated by the Somogyi–Nelson method[20] and a value of 16200 was obtained. OPS was composed predominantly of mannose with small amounts of hexosamine and KDO (Table II). When the ultraviolet absorption spectrum of OPS was recorded at various concentrations in aqueous solution, there were no absorption bands at 260 and 280 nm, indicating that no appreciable amounts of nucleic acids and proteins are present, although qualitative analysis by the method of Lowry[16] showed the presence of 0.14% protein. In the infrared spectrum of OPS, no significant absorption was observed in the vicinity of 1725 or 1250 cm^{-1}, corresponding to *O*-acyl groups. Moreover, the type IIb absorption band near 890 cm^{-1} [21] was not observed in its infrared spectrum. The specific optical rotation of OPS was $[\alpha]_D^{25}$ $+88°$ ($c=0.5$, in water). These results suggest that the D-mannose residues of OPS have the α-configuration.

Methylation Analysis

Determination of glycosidic linkages of D-mannose residues of OPS was carried out by methylation analysis. OPS was methylated three times; the fully methylated polysaccharide thus obtained showed no absorbance at 3400—3500 cm^{-1} due to hydroxyl groups in the infrared spectrum, indicating that methylation was complete. The partially methylated alditol acetates were subjected to GC-MS. In the GC, three peaks were detected (Fig. 3) and were identified from the mass fragmentation patterns on GC-MS, as previously reported.[22] The

ratio of the peak areas of the two major components was calculated to be 1.56 : 1.00 (II : III). MS analysis showed that the MS of peak I contained fragments having the main mass numbers (*m/e*) 45, 89, 117, 129, 161 and 205. The MS of peak II showed fragments having *m/e* 45, 87, 101, 129, 161 and 189, and that of peak III showed fragments having *m/e* 45, 101, 117, 129, 161 and 233. From these results, peaks I, II and III were identified as 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylmannitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylmannitol respectively. The molar ratio of peaks II and III was 3 : 2. There was no peak corresponding to di-*O*-methylmannose, indicating that OPS has no branched structure.

Smith Degradation

About 38% of mannose residues was found to be resistant to periodate oxidation. The polysaccharide was oxidized with periodate, reduced with $NaBH_4$, and then completely hydrolyzed with 1 N H_2SO_4. Analysis of the product by TLC revealed unoxidized mannose and glycerol. These results suggest that OPS is composed of (1→2)-linked mannopyranose residues, which produce glycerol upon periodate oxidation followed by borohydride reduction and (1→3)-linked mannopyranose residues which are not oxidized. Moreover, these results indicate that about three-fifths of the mannose is substituted at position 2 and about two-fifths is substituted at position 3. The results of analysis of Smith degradation were in good agreement with those of the methylation analysis. In addition, the oxidized and reduced polysaccharide polyalcohol (10 mg) was subjected to mild acid hydrolysis, and the hydrolyzate was methylated and then hydrolyzed with acid, reduced with borohydride, and acetylated as described in Experimental. The partially methylated alditol acetates thus obtained were subjected to GC-MS. As shown in Fig. 4, two major peaks were detected and were identified from the mass fragmentation patterns on GC-MS. Peaks I and III corresponded to 2,3,4,6-tetra-*O*-methylmannitol and 2,4,6-tri-*O*-methylmannitol indicating that they were due to non-reducing terminal mannose and mannose residues substituted at position 3, respectively. This suggests that Smith degradation and partial acid hydrolysis of OPS liberated mannosyl-(1→3)-mannose.

Discussion

Our earlier studies have shown that the neutral polysaccharide isolated from culture supernatant of *Klebsiella pneumoniae* strain Kasuya (O3 : K1) or its decapsulated mutant strain LEN-1 (O3 : K1$^-$) exhibits an extraordinarily strong adjuvant action on both antibody response[1-4,8] and delayed-type hypersensitivity[23,24] to various antigens in mice. Further studies have revealed that the neutral polysaccharide isolated from the decapsulated mutant strain LEN-1 possesses the same strong adjuvant activity as that isolated from the parent strain, and that the substance is antigenically identical to the O3 antigen of *Klebsiella* and the O9 antigen of *E. coli*.[9]

In the present study, we analyzed the O3 antigen isolated from culture supernatant of the decapsulated mutant strain LEN-1. It was found that the O3 antigen consisted of 59—66% carbohydrates and 25—27% lipids. This shows that the chemical nature of the O3 antigen from culture supernatant of the decapsulated mutant strain LEN-1 is LPS. The results of analysis of carbohydrate compositions of OPS are consistent with those of our previous study[25] and indicate that it consists predominantly of D-mannose. In order to elucidate the chemical structure of OPS, we analyzed the acid hydrolysis products obtained from the fully methylated polysaccharide by GC-MS. The results showed that the mannan contained (1→2)- and (1→3)-linkages in a molar ratio of 3 : 2. For the structure of the mannan, two alternatives may be considered: a random distribution of (1→2)- and (1→3)-linkages in the polysaccharide

chain, and an ordered sequential structure consisting of repeating units. In this study, Smith degradation and partial acid hydrolysis of OPS liberated mannosyl-(1→3)-mannose. On the basis of this result, it is most likely that OPS consists of a mannan which has units of α-mannosyl-(1→3)-α-mannosyl-(1→2)-α-mannosyl-(1→2)-α-mannosyl-(1→2)-α-mannose joined through α-mannosyl-(1→3)-linkages.

The polysaccharide moiety of the O3 antigen isolated from bacterial cells of *Klebsiella*[26] and that of the O9 antigen isolated from the cells of *E. coli*[27] consisted of a mannan which has the same structure as proposed for OPS in the present study. Moreover, it has been found that the O3 antigen of *Klebsiella* and the O9 antigen of *E. coli* are serologically identical.[28] Prehm *et al.*[27] reported that the optical rotations of the polysaccharides from *E. coli* O9 and from *Klebsiella* O3 were identical, $[\alpha]_D^{25} = +89^\circ$. The optical rotation of OPS presented in this report ($+88^\circ$) is in good agreement with this value. In addition, OPS showed a single peak in gel filtration analysis, and gave molecular weight values of 14000 and 16200 as determined by gel filtration and by the Somogyi–Nelson method, respectively. These are very similar to the value (12500) found for the polysaccharide moiety of *E. coli* O9 by ultracentrifugal analysis.[27] On the other hand, the polysaccharide moiety of the O5 antigen isolated from the bacterial cells of *Klebsiella* by Lindberg *et al.*[29] consisted of a mannan which has units of α-mannosyl-(1→2)-α-mannosyl-(1→3)-α-mannosyl-(1→2)-α-mannosyl-(1→2)-α-mannose joined through α-mannosyl-(1→3)-linkages.

The antigenicity of the O3 antigen isolated from culture supernatant of the decapsulated mutant strain LEN-1 is not identical to that of the O5 antigen of *Klebsiella*, and the optical rotations of the polysaccharides from *Klebsiella* O3 and from *Klebsiella* O5 ($+42^\circ$) are not identical.[29]

If the structure of the repeating unit of OPS postulated in the present study is valid, the mean molecular weight of OPS (about 15000) and the content of mannose (92.4%) in OPS suggest that OPS consists of 17 repeating units. The preparation of OPS employed in the present study contained small amounts of hexosamine and KDO, which were probably components of the core oligosaccharide. The precise structure of the core oligosaccharide is not yet known. Some physico-chemical properties and the chemical structure of the core oligosaccharide will be reported in the next paper of this series.

References

1) I. Nakashima, *J. Immunol.*, **108**, 1009 (1972).
2) I. Nakashima and N. Kato, *Immunology*, **27**, 179 (1974).
3) I. Nakashima, T. Kobayashi, and N. Kato, *J. Immunol.*, **107**, 1112 (1971).
4) I. Nakashima, F. Ohta, T. Kobayashi, O. Kato, and N. Kato, *Immunology*, **26**, 443 (1974).
5) I. Nakashima, F. Nagase, A. Matsuura, and N. Kato, *Cell. Immunol.*, **49**, 360 (1980).
6) I. Nakashima, F. Nagase, T. Yokochi, M. Ohta, and N. Kato, *Cell. Immunol.*, **46**, 69 (1979).
7) I. Nakashima and N. Kato, *Jpn. J. Microbiol.*, **17**, 461 (1973).
8) M. Ohta, M. Mori, T. Hasegawa, F. Nagase, I. Nakashima, S. Naito, and N. Kato, *Microbiol. Immunol.*, **25**, 939 (1981).
9) N. Kato, M. Ohta, T. Hasegawa, M. Mori, K. Yamaki, K. Mizuta, I. Nakashima, and S. Naito, *Microbiol. Immunol.*, **25**, 1317 (1981).
10) T. Hasegawa, M. Ohta, M. Mori, I. Nakashima, and N. Kato, *Microbiol. Immunol.*, **27**, 683 (1983).
11) A. B. Batshon, H. Baer, and M. F. Sheffer, *J. Immunol.*, **90**, 121 (1962).
12) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
13) A. Nowotny, "Basic Exercises in Immunochemistry," Springer-Verlag, Berlin, 1969, pp. 97—99.
14) V. A. Waravdekar and L. D. Saslaw, *J. Biol. Chem.*, **234**, 1945 (1959).
15) C. J. M. Rondle and W. J. J. Morgan, *Biochem. J.*, **61**, 586 (1954).
16) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
17) W. E. Trevelyan, D. P. Procter, and J. S. Harrison, *Nature* (London), **166**, 444 (1950).
18) S. Hakomori and T. Ishimoda, *J. Biochem.*, **55**, 205 (1964).

19) F. Smith and J. W. Van Cleve, *J. Am. Chem. Soc.*, **77**, 3091 (1955).
20) M. Somogyi, *J. Biol. Chem.*, **195**, 19 (1952).
21) S. A. Barker, E. J. Bourne, and D. H. Whiffen, "Methods of Biochemical Analysis" ed. by D. Glick, Interscience Publishers, Inc., New York, 1956, p. 213.
22) H. Bjorndal, C. G. Hellergvist, B. Lindberg, and S. Svensson, *Angew. Chem.*, **82**, 643 (1970).
23) M. Ohta, I. Nakashima, and N. Kato, *Immunology*, **163**, 460 (1982).
24) M. Ohta, I. Nakashima, and N. Kato, *Cell. Immunol.*, **66**, 111 (1982).
25) M. Ohta, M. Mori, I. Nakashima, and N. Kato, *Microbiol. Immunol.*, **23**, 805 (1979).
26) M. Curvall, B. Lindberg, J. Lönngren, and W. Nimmich, *Acta Chem. Scand.*, **27**, 2645 (1973).
27) P. Prehm, B. Jann, and K. Jann, *Eur. J. Biochem.*, **67**, 53 (1976).
28) F. Kauffmann, *Acta Microbiol. Scand.*, **26**, 381 (1949).
29) B. Lindberg, J. Lonngren, and W. Nimmich, *Acta Chem. Scand.*, **26**, 2231 (1972).