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Application of Optical Properties of the Vi Capsular Polysaccharide for Quantitation of the Vi Antigen in Vaccines for Typhoid Fever

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The capsular polysaccharide of *Salmonella typhi* and of *Citrobacter freundii* (Vi) is a linear homopolymer of α1,4-linked *N*-acetylgalactosaminuronic acid, variably O-acetylated at the C-3 position. Vaccines composed of Vi confer protection against typhoid fever with an efficacy of about 70%; Vi has recently been conjugated to proteins to increase its immunogenicity and effectiveness (I. L. Acharya, R. Tapa, V. L. Gurubacharya, M. B. Shrestha, C. U. Lowe, D. D. Bryla, R. Schneerson, J. B. Robbins, T. Crampton, B. Trollfors, M. Cadoz, D. Schulz, and J. Armand, N. Engl. J. Med. 317:1101–1104, 1987; K. P. Klugman, I. Gilbertson, H. J. Kornhof, J. B. Robbins, R. Schneerson, D. Schulz, M. Cadoz, and J. Armand, Lancet ii:1165–1169, 1987; S. C. Szu, A. L. Stone, J. D. Robbins, R. Schneerson, and J. B. Robbins, J. Exp. Med. 166:1510–1524, 1987). Vi, however, cannot be measured by conventional colorimetric methods. Two optical techniques were adapted to quantitate Vi in vaccines. The first, Fourier-transformed infrared spectroscopy, was performed on salt-free, freeze-dried samples. The intensities of the absorbance peaks of Vi were proportional to the amount of Vi within the range of 0.25 to 2.0 mg. The amount of Vi was determined from integrated absorptions at the 1,235- or 1,417-cm^{-1} band. The second technique, spectrophotometric titration, was more sensitive than the Fourier-transformed infrared spectroscopy and could be performed on dilute solutions. The metachromatic effect of the reaction between the aromatic cationic dye acridine orange and the carboxyl groups of Vi was quantitative within ±2% in the range of 20 to 700 μg of Vi per ml. The accuracy of the titration of Vi in the vaccines was within ±8%. These two methods may be applicable to measure other capsular polysaccharides in vaccines.

Capsular polysaccharides (CPS) are essential for the invasiveness of capsulated bacteria (8). Vaccines composed of CPS induce immunity in adults and older children by their ability to elicit serum antibodies by "T-independent" mechanisms (5, 9, 11, 17–19, 25). Through the preparation of CPS-protein conjugates, it is anticipated that the antibody response to CPS will be enhanced and that protection against diseases caused by these pathogens will be extended to infants and to adults with immunodeficiencies (4, 21–24, 36). Investigation of the physicochemical properties of CPS may provide insights into their virulence-promoting and immunity-inducing roles.

The CPS of *Salmonella typhi* (Vi) is a linear homopolymer of a hexosaminuronic acid, α1,4-*N*-acetylgalactosaminuronic acid, variably O-acetylated to about 90% at the C-3 position (Fig. 1) (2, 6, 7, 34–36). The Vi vaccine conferred protection against typhoid fever in two clinical trials (1, 12a). Standardization of vaccines composed of Vi or of newly developed Vi-protein conjugates has been hindered for want of a method for quantifying Vi. Colorimetric methods for measuring amino sugars or uronic acids are not applicable to the measurement of Vi because the polyhexosaminuronic acid structure is resistant to acid hydrolysis and the aminouronic acid moieties do not form the chromophore in the carbazole assay (2, 3, 26, 34, 35, 37).

Vi resembles mammalian glycosaminoglycans in having repeat units that consist of sugars with both *N*-acetyl and uronic acid groups. Hexosaminuronic acids, although uncommon in nature, have been found in other bacteria, e.g., the CPS of some *Staphylococcus aureus* strains, the acidic fibrous polysaccharide of *Achromobacter georgiopolitanum*, *Streptococcus pneumoniae* type 12F, *Haemophilus influenzae* types d and e, and *Escherichia coli* K7 (8, 10, 12, 13–15, 26, 37).

This report describes two methods for quantitating Vi or Vi covalently bound to proteins (conjugates) by physicochemical methods that do not rely on depolymerization of the polysaccharide. First, Fourier-transformed infrared (FTIR) spectroscopy was applied to dried samples of Vi and Vi conjugates. Second, the carboxyl groups of Vi alone or as a component of a conjugate were quantitated in dilute solution by spectrophotometric titration with the aromatic cationic dye acridine orange (AO), adapted from techniques developed to measure glycosaminoglycans and other acidic polysaccharides (31).

MATERIALS AND METHODS

Vi and Vi-protein conjugates. Vi was purified from *Citrobacter freundii* WR7011 (courtesy of Louis Baron, Walter Reed Army Institute of Research) as described previously (31a, 36); final products contained less than 1% lipopolysaccharide or protein and less than 2.0% nucleic acids (31a, 33). Elemental analysis of preparation 1 showed 0.33% P, 3.71% N, 5.09% Ca^{2+}, and 10.1% moisture; analysis of preparation 2 showed 0.09% P, 6.87% N, 0.05% Ca^{2+}, and 8.64% moisture. The molecular weight was estimated to be 3.5×10^6 by gel filtration through Sepharose CL-4B with Dextran T-2000 (Pharmacia, Inc., Piscataway, N.J.) as a reference. Vi conjugates were synthesized with proteins derivatized with the heterobifunctional reagent *N*-succinimidyl 3-(-2-pyridyldithio)propionate and thiol derivatives of the Vi (31a). About 1 in every 55 residues of Vi was thiolated. The

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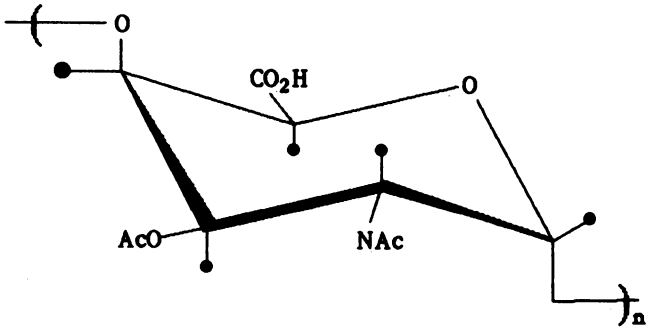

FIG. 1. Repeating structure of the *S. typhi* and *C. freundii* CPS (Vi) is an α1,4-linked *N*-acetylgalactosaminuronic acid, O-acetylated up to 90% at the C-3 position (2, 6, 7, 34). These glycosidic bonds are all axial-axial linkages, which form an intercharge distance of ~0.43 nm in similar biopolymers (1a, 16). NAc, *N*-acetyl; AcO, O-acetyl; ●, hydrogen.

proteins were cholera toxin (CT), the B chain of cholera toxin (CTB), tetanus toxoid (TT) (Institut Merieux, Lyon, France), diphtheria toxoid (DT) (Instituut voor Volksgezonheid, Bilthoven, The Netherlands), and bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.). DT, after passage through S-300 Sephacryl to remove polymerized and degraded toxoid, was light brown, presumably owing to components of the Formalin-treated medium.

Hib. *H. influenzae* type b polysaccharide (Hib) and a conjugate of Hib with TT (Hib-TT) were kindly supplied by Rachel Schneerson (22).

FTIR spectroscopy. All samples were equilibrated against glass-distilled water (GDW) by extensive dialysis. Sample disks were pressed from freeze-dried solutions of Vi (0.5 to 2.0 mg), proteins (0.8 to 1.2 mg), or Vi conjugates (1.0 to 2.9 mg) that were added to 100 mg of KBr and then dissolved in 2.0 ml of GDW. FTIR spectra (500 scans) of the disks were recorded in a Nicolet 1179 spectrometer (2-cm^{-1} resolution) and processed in a Nicolet 1180 data system. Overlapping absorption peaks were resolved by fitting absorption bands to Gaussian curves. The amount of Vi or protein was calculated from the area under the Gaussian curve by comparison with that of a standard. The FTIR absorption at the amide bands of proteins (1,650 and 1,540 cm^{-1}) was used to quantitate the carrier proteins. Vi was measured from the intensity of its absorption bands at 1,650, 1,604, 1,417, and 1,235 cm^{-1}. The amount of Vi in the conjugates was determined from the absorption intensity of the conjugate at 1,235 or 1,417 cm^{-1}. The FTIR spectrum of this amount of Vi (computed from a standard spectrum) was then subtracted from that of the Vi conjugate. The amount of protein in the conjugate was calculated from the intensity of the 1,540-cm^{-1} absorption band in the resultant spectrum.

Spectrophotometric titration. Aromatic cationic dyes exhibit metachromatic reactions upon binding to acidic polysaccharides that have less than 0.7 nm between their negative charges (27, 30, 31). This proximity permits optical interactions between bound neighboring dyes. Several changes occur in the absorption spectrum of the dye owing to these dye-dye interactions. One optical effect is the frequency shift of the visible absorption band (color change). Another is hypochromism. The intensity of the absorption is decreased by the borrowing of energy from the visible to the UV absorption band of the dye (27). With a constant amount of dilute dye, both the frequency shift and the hypochromism are proportional to the amount of acidic polysaccharide added. Strong metachromatic reactions occur with acidic polysaccharides that have a charge density of 0.43 to 0.51 nm (about 1 per pyranose monosaccharide), such as Vi (27, 31). The hypochromism and frequency shift that occur when the dyes react with acidic polysaccharides provided the basis for the spectrophotometric titration of Vi.

Recrystallized AO (National Aniline, Chicago, Ill.) and methylene blue (MB; Chroma-Gesellshaft, Roboz Surgical Co., Washington, D.C.) were prepared as stock solutions, 1.7×10^{-4} and 2.2×10^{-4} M, respectively, in 10 mM sodium cacodylate buffer (pH 6.8). Samples of the stock solutions were added to GDW in a glass-stoppered optical cell (QS grade; Opticell, Kensington, N.J.) to prepare the dilute solutions of the dye for spectrophotometric titration (~1.7×10^{-5} M AO or ~1.2×10^{-5} M MB) (31).

The pHs of the dilute AO solutions, initially 6.6 to 6.8, were 6.2 to 7.1 at the end of the titrations. Since it was necessary to maintain the pH sufficiently high to ensure that all the carboxyl groups of Vi remained unprotonated, samples were titrated in parallel with either 0.3 mM $NaHCO_3$ or GDW as the dye diluent. The calculated values of Vi in the paired samples were indistinguishable. Accordingly, GDW was used in all ensuing titrations. Stock dye solutions kept air tight in the dark at 4 to 8°C were stable for several years.

Samples of Vi were added serially to the diluted AO solution with a Hamilton microsyringe and stirred for about 2 min with a micro stirring bar. The absorption of the AO solutions was recorded between 530 and 430 nm on a Cary 118C spectrophotometer; MB solutions were scanned between 700 and 430 nm. With the excess of dye, increments of Vi gave a linear decrease in the extinction coefficients of AO (E_{490}) and of MB (E_{665}). This constituted the descending limb of the titration (31). With excess Vi, the E_{490} remained constant upon additional increments, forming the second (horizontal) limb. At the intersect of the two limbs (point of equivalence), the nanomoles of the reactive unit of the carboxyl groups equalled the nanomoles of dye. Glycosaminoglycans at concentrations of 300 to 1,000 μg/ml gave the standard titration plot with a reproducibility of about 3% (31). For Vi, a concentration range of 200 to 600 μg/ml could be assayed by this spectrophotometric titration. Concentrations of >700 μg of Vi per ml necessitated small volumes of addition (1 to 2 μl), slower delivery of the Vi from the 10-μl Hamilton syringe because of viscous drag, and longer periods of stirring.

CD spectroscopy. The low UV circular dichroism (CD) spectrum of Vi was measured in a Cary model 60 spectropolarimeter as described for glycosaminoglycans (28–30). Spectra were taken from 300 to 188 nm, with a light path of 0.5 mm and a Vi concentration of 1.2 mg/ml (300 to 200 nm) or 0.6 mg/ml (210 to 188 nm). Preliminary UV CD spectra of Vi (not shown) exhibited two peaks in the 300- to 188-nm range, a strong positive band at ~194 nm that overlapped one or more weaker bands around 215 nm. The former would be expected from the *N*-acetyl group of an α1,4-linked amino sugar; the latter can be attributed to the carboxyl substituent as well as the *N*-acetyl group of the repeating sugar (Fig. 1) (28, 30). The molar residue ellipticity at 194 nm (ellipticity in degrees $centimeter^{-2}$ decimole of $monosaccharide^{-1}$) was ~37,000, suggesting that quantitative measurement of the unconjugated Vi was possible from its CD absorption spectrum.

RESULTS

FTIR analyses. FTIR spectra of Vi and CT are illustrated in Fig. 2A and B. The amide bands of the CT (1,650 and

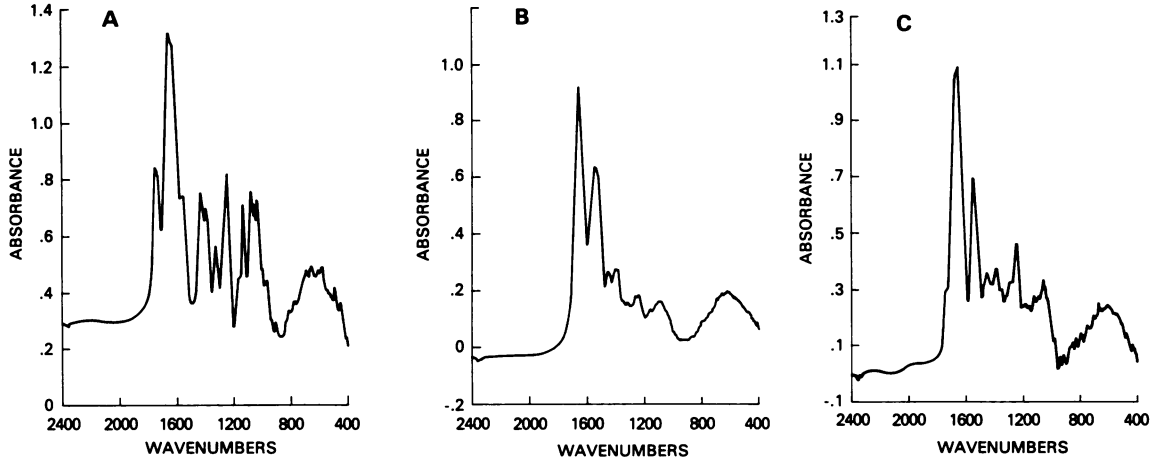

FIG. 2. FTIR spectrum of Vi polysaccharide (1.4 mg in 102 mg of KBr) (A), CT (1.0 mg in 99 mg of KBr) (B), and Vi-CT conjugate (1.2 mg of conjugate in 101 mg of KBr) (C); the ratio of protein to Vi is 0.38.

1,540 cm^{-1}) are prominent (Fig. 2B). Infrared (IR) bands of Vi (Fig. 2A) were assigned to the various substituents of the CPS: *O*-acetyl and/or protonated carboxyl (1,740 cm^{-1}), carboxylate anion (1,604 and 1,417 cm^{-1}), *N*-acetamido (1,650 and 1,540 cm^{-1}), and acetic esterlike —C—O— (1,235 cm^{-1}) (32). Most of the IR bands of Vi overlapped either another Vi band or a protein band. The IR peak of Vi at 1,235 cm^{-1}, however, was well separated from the other peaks. FTIR absorption peaks were analyzed as a function of various amounts of Vi. The intensities of four of the peaks were approximately linear within the range of 0.25 to 2.0 mg of Vi (Fig. 3). The averages of these four FTIR peak intensities at each amount of Vi (1.43, 0.66, 0.28, and 0.16) were linear with the Vi amounts of 2.0, 1.0, 0.5, and 0.25 mg, paired respectively. The average deviation of these paired values from linearity was 9%.

The FTIR spectrum of Vi-CT_{VII} is shown in Fig. 2C. Contributions from both Vi and the protein are observed in the 1,650- and 1,540-cm^{-1} bands of the conjugate. The amount of Vi was determined from its FTIR absorption at 1,235 cm^{-1} by comparison with a standard. The amount of protein in the conjugate was then determined by the intensity of the 1,540-cm^{-1} absorption band after correction for the absorbance of the Vi (Materials and Methods). The 1,540- and 1,235-cm^{-1} bands were resolved from overlapping absorption bands by fitting the peaks to Gaussian curves. FTIR analyses of the Vi and protein were used to standardize experimental vaccines composed of Vi-CT, Vi-TT, Vi-DT, and Vi-bovine serum albumin. FTIR spectra of the Vi-DT conjugates, however, exhibited rising base lines in the low-wave-number region. In such cases, correction of the base line could decrease the accuracy of the measurement of Vi.

Standard titration and stoichiometry. On the basis of the intersugar distance of other axially linked 1,4-D-galactose polysaccharides (determined to be 0.43 nm), we reasoned that dyes bound consecutively to carboxyl groups of Vi would be close enough to undergo strong metachromatic coupling and provide a spectrophotometric analysis of this CPS (1a, 16, 27). Addition of Vi to the dilute solutions of AO or MB promoted strong hypochromism and a moderate shift in frequency (Fig. 4A and B). The metachromatic hypochromism of AO bound to Vi is depicted in Fig. 4, inset. The descending limb of the titration was linear and gave a sharp endpoint at the intersect with the horizontal limb. At this endpoint, the nanomoles of the dye-binding units of Vi equal the nanomoles of AO. The ratio of AO to the carboxyl groups at the endpoint was determined for several samples of Vi from two preparations and a concentration range of 0.4 to 1.2 mg/ml. The stoichiometry of the standard titration was close to one AO per carboxyl group (Table 1).

The concentrations of Vi calculated from these titrations were within 85 to 104% of that expected from the dry weight (Table 1, columns 4 and 2). The average value and standard deviation of the ratio of AO to carboxyl groups (AO/COO^-) among the various samples was 0.92 ± 0.08. At concentrations above 1 mg of Vi per ml, one-to-one binding of AO to the carboxyl groups appeared to diminish by several percent. This concentration of Vi may be too high for quantitation because of its viscosity and small volume of additions (~1 μl).

MB also exhibited strong metachromatic reactions and about one-to-one stoichiometry when bound to Vi (data not shown). AO was chosen for quantitation of the Vi vaccines

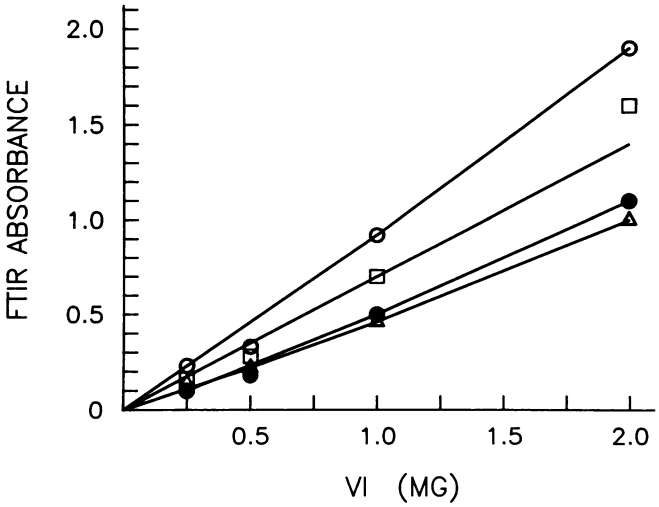

FIG. 3. FTIR absorption versus amount of Vi polysaccharide. The relative intensities at the 1,650 (○), 1,604 (□), 1,417 (△), or 1,235 (●) cm^{-1} band of Vi for 2.0, 1.0, 0.5, and 0.25 mg were 1.9, 0.92, (0.46), 0.23; 1.6, 0.75, 0.28, 0.16; 1.0, 0.46, 0.22, 0.14; and 1.1, 0.50, 0.18, 0.10, respectively. The value in parentheses was taken from the average straight line.

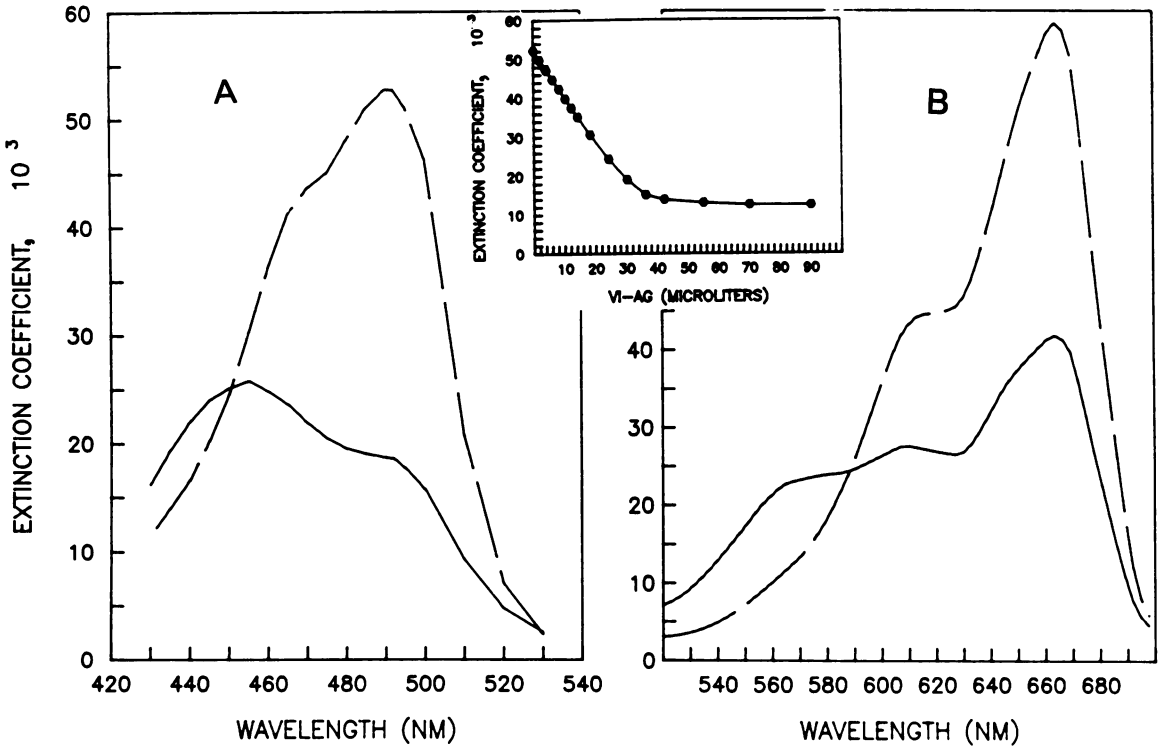

FIG. 4. Metachromatic reactions of AO (A) and MB (B). The spectrum of the free monomeric dye (– – –) is contrasted with the metachromatic spectrum of the dye when bound to the carboxyl groups of the Vi (——). The concentrations of the standard solutions of AO and MB diluted in GDW were 1.67×10^{-5} and 1.2×10^{-5} M, respectively. Inset: The hypochromic change in extinction coefficient of AO at 490 nm is plotted as a function of serial additions of a solution of preparation 1 (540 μg of Vi per ml of GDW). The endpoint of the titration is the intersect of the two linear limbs. AG, Antigen.

in subsequent studies because of its stronger binding affinity (31).

A series of dilutions, 16.2 to 328 μg of Vi per ml, made from a sample of Vi were titrated with AO. The reaction was linear through this range (Table 1), and the lower limit of the titration was 16 μg of Vi per ml. The average value and standard deviation of AO/COO^- in the titrations was 1.02 ± 0.02.

Titration of dilute Vi solutions. The large volumes of additions that would be necessary for low concentrations of acidic polysaccharides could engender errors owing to a change in the extinction of the dye or a decrease in the sharpness of the endpoint. Modification of the standard titration was necessary, therefore, to measure concentrations of Vi in vaccines in the range of ~40 to 200 μg/ml. First (extrapolation method), when endpoints were <100 μl, the linear portion of the descending limb could be extrapolated to intersect with a horizontal limb drawn at E = 12,400 (the average minimum extinction of the AO-Vi complexes). Second (slope method), for concentrations of <100 μg of Vi per ml, it was necessary to calculate the endpoint from the initial slope, S, of the descending limb. S was determined by the addition of up to 60 μl of Vi in 10- or 20-μl increments, where

$$S = \text{microliters added/change in } E_{490} \text{ at each point} \quad (1)$$

$$\text{microliters at endpoint} = kS \quad (2)$$

and $k = E_{490}$ of free AO (52,000) − minimum E_{490} (12,400) = 39,600. As in the standard titration, the number of microliters at the endpoint yields the concentration of Vi (Table 1).

TABLE 1. Spectrophotometric titration of carboxyl groups of Vi by AO; concentration range and stoichiometry[a]

Titration (prepn and sample no.)	μg of Vi/ml	mM COO^- (nmol of AO/μl of Vi)[b]	μg of Vi/ml (calculated)[c]	AO/COO^-[d]
Standard				
1 (1)	1,240	3.80	1,050	0.85
1 (2)	540	1.70	471	0.87
1 (3)	328	1.21	334	1.02
2 (1)	1,035	3.08	852	0.82
2 (2)	55	0.195	57	1.04
Standard and modified				
1 (3)	328	1.21	334	1.02
1 (3)	162	0.614	169	1.04
1 (3)	81	0.294	81	1.00
1 (3)	32.8	0.118	32.7	1.00
1 (3)	16.2	0.0611	16.8	1.04

[a] Results with different preparations and various samples are listed in the upper part of the table. The stoichiometry for preparations 1 and 2 was close to 1.0, and the reproducibility of AO/COO^- among the samples was 0.92 ± 0.08. Results with dilutions from the same sample are listed in the lower part of the table; reproducibility of AO/COO^- over the 20-fold concentration range of these titrations was 1.02 ± 0.02.

[b] Concentration of Vi (millimolar COO^-) determined by the known amount of AO (41 to 42 nmol) per microliter of Vi at the equivalence point.

[c] Micrograms of Vi per milliliter (calculated) = value in column 3 × 277 (equivalent weight based on 90% O-acetylation).

[d] AO/COO^- = nanomoles of AO/nanomoles of COO^- at endpoint (based on value in column 2/equivalent weight).

Finally, a third modification permitted direct determination of the concentration of Vi from a standard plot of $1/S$ versus micrograms of Vi per milliliter (Fig. 5). This standard plot was derived from the data in Table 1 and can be applied to the measurement of Vi, provided that the conditions of concentration, total nanomoles, and E_{490} of AO are the same as in Fig. 5.

Effect of vaccine solvent. One difficulty in adapting this spectrophotometric titration to the measurement of Vi in vaccines was the physiological saline (0.15 M NaCl) required for the solvent. Spectrophotometric analysis of other acidic polysaccharides with AO was unaffected by ionic strengths up to ~3 mM (A. L. Stone, unpublished data). At higher concentrations of NaCl, however, the binding of cationic dyes such as AO to polyanions is inhibited. Standard titrations of more than 200 μg of Vi per ml would be unaffected by saline as the solvent because the ionic strength at the endpoint would remain <3 mM. Less than 200 μg of Vi per ml in saline could be quantitated by the above modifications for dilute solutions. The sensitivity of this assay (sufficient hypochromism to yield a valid slope) was about 40 to 50 μg of Vi per ml of saline. Representative paired titrations of Vi dissolved in saline or GDW demonstrated a negligible effect exerted by the saline on the initial slope (within 5%) (Table 2). Identical data were obtained when the extinction of the titration was recorded before and after the addition of an equivalent amount of saline. Table 2 also illustrates that the presence of DT in the relative amount contained in the conjugates did not interfere with the titration of Vi.

Quantitation of Vi in conjugates. Analyses of Vi were regularly achieved for Vi-CT and Vi-CTB conjugates in saline by the slope or extrapolation method (see above). The linearity of the hypochromism of AO with a representative conjugate, Vi-CT_{xii}, compared with that of Vi alone is shown in Fig. 6. The amount of Vi determined by this titration was in agreement with the value obtained by FTIR. Vi-CT_{xii} contained 54 μg of Vi per ml by FTIR and 41 and 43 μg of Vi per ml by spectrophotometric titration (extrapolation and slope methods, respectively). The difference in Vi obtained by the two methods is expected because of two factors: (i) derivatization of the Vi by forming an amidelike bond with cystamine would reduce both the number of carboxyl groups interacting with AO and the resultant hypochromism generated by AO-AO interaction; (ii) covalent association of Vi with the protein could reduce the AO binding and hypochromism through steric effects. Differences between the results obtained by spectrophotometric and FTIR determinations (20%) could be reduced by assuming that about 10% of the Vi had been rendered nonmeasurable with AO as a result of the conjugation procedure.

The extent of the linearity of hypochromism of AO and other conjugates did not always allow reliable determination

TABLE 2. Effect of vaccine solvent or protein on the spectrophotometric titration of Vi

Sample[a]	Vi solvent	nmol of AO	AO diluent	Endpoint (μl of AO)	μg of Vi/ml	Relative percentage[b]
2	GDW	40.68	GDW	292	38.3	100
2	SS	40.68	GDW	310	36.1	94
3	GDW	41.64	GDW	353	32.7	100
3	SS	40.74	GDW	331	34.1	104
2	GDW	41.00	B[c]	24.0	474	100
2	GDW	41.55	B + DT	26.1	449	94

[a] Samples were from preparation 1; the Vi sample was in GDW or in 0.15 M NaCl (SS). Micrograms of Vi per milliliter was calculated as described in Table 1, footnote *c*.

[b] Values obtained by varied titrations relative to those obtained under standard conditions.

[c] B, 0.3 mM bicarbonate, pH 7.5.

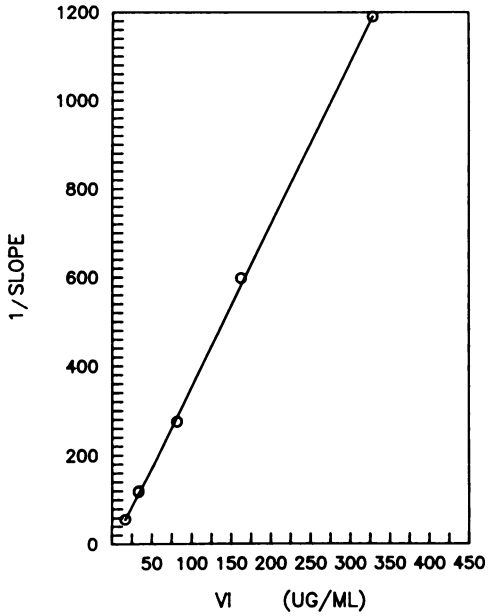

FIG. 5. Reciprocal of the initial slope of the descending limb of the AO titration versus the concentration of Vi polysaccharide. The initial slope of the titration of dilute Vi (<100 and >20 μg of Vi per ml of GDW) was obtained by the addition of up to 60 μl of sample in 10- or 20-μl aliquots. The diluted dye solution contained 42 nmol of AO in 2.4 ml of GDW, and the E_{490} was 52,200.

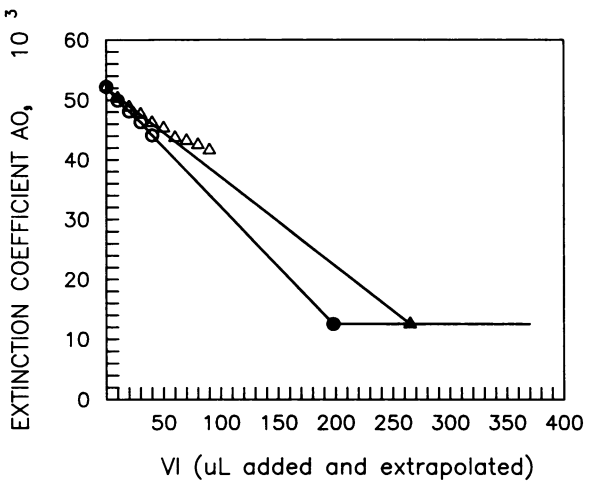

FIG. 6. Determination of Vi in conjugates by the extrapolation or slope method. A solution of Vi (54 μg/ml of 0.15 M NaCl) (○) or of Vi-CT (54 μg of Vi per ml of 0.15 M NaCl) (△) (by FTIR analysis) was added in 10-μl aliquots to the dilute AO solution (42.0 nmol of AO). The initial linear portion of the descending limb of the titration could be extrapolated to the horizontal (●, ▲) or could yield the initial slope to obtain the concentration of Vi. The value thus obtained for the Vi sample was 54 μg of Vi per ml. Values for the sample of Vi-CT were 41 and 43 μg of Vi per ml by the extrapolation and slope methods, respectively.

of Vi by the slope method. For example, several Vi-DT conjugates in 0.2 M NaCl yielded nonlinear initial slopes for the determination of Vi, which were not improved by increasing the time of stirring or standing. The concentration of Vi in these samples was close to the lower limit of the assay (~40 μg/ml), and the errors in measurement of Vi were as high as ±20%. A preparation of Vi-TT exhibited a similar nonlinearity in the initial slope.

Agreement between spectrophotometric titration and colorimetric measurements. The results of AO titration could not be compared with those of a colorimetric assay of Vi. That the AO method was in agreement with a conventional chemical assay was validated by using an acidic CPS that is standardized by the Bial reaction (22); the CPS of *H. influenzae* type b (Hib) elicits protective antibodies and has one PO_4^- in its disaccharide repeat [3-D-ribosyl furanose-(1-1)-ribitol-5-PO_4-] (12, 22, 25). The metachromatic reaction of AO with Bial-standardized solutions of Hib (1,000 and 400 μg/ml) in GDW gave a linear hypochromic limb; the concentrations of Hib thus obtained were in agreement within experimental error (1,040 and 390 μg/ml, respectively) (A. L. Stone and R. Schneerson, unpublished data). The same agreement was demonstrated when the CPS was measured as a component of the Hib-TT conjugate in 0.15 M NaCl by the AO slope method (k = 32,200 in equation 2).

DISCUSSION

Two clinical trials have established that Vi vaccines confer protective immunity against typhoid fever (1, 12a). Protein conjugates of Vi were developed to increase the immunogenicity, and thereby the effectiveness, of the Vi vaccines (31a). Since Vi could not be measured by colorimetric assays, alternative methods adapted from FTIR spectroscopy and spectrophotometric titration with AO were established to quantify the Vi in vaccines.

The FTIR spectroscopic method quantified both the Vi or the Vi and protein in conjugates as salt-free solids. Certain absorption bands of conjugates contained intensity contributions from Vi only; IR vibrational signals from peptide bonds, rather than analysis of individual amino acids, were used to quantitate the protein. The composition of the conjugates was obtained by determining the individual contributions of Vi and protein to the intensities of these absorption bands. The Vi-DT conjugates exhibited a relatively high background in the low-wave-number portion of the spectrum. One explanation for this could be the inhomogeneity of DT, which is a Formalin-treated protein.

Measurement in solution was achieved by quantitating the metachromasia induced by the binding of AO to the carboxyl groups of Vi; the stoichiometry was one AO per COO^-. The sharpness of the endpoint in the standard plot substantiated that the anionic density of the Vi was evenly distributed throughout the polymer. As low a concentration as 20 μg of Vi per ml could be measured in GDW. The reproducibility was ±2% for the same sample and ±8% among different preparations or samples of Vi. This variation might be anticipated considering the inhomogeneities in salt and/or moisture that may occur in preparations of high-molecular-weight biopolymers.

Spectrophotometric analyses of Vi by the slope method could be also applied to conjugates. The Vi in the conjugate reacted like the native polysaccharide. For example, the stoichiometry between the Vi-CT conjugate and AO was ~0.9 AO per monosaccharide (corrected for the ~10% of the residues that would be undetected by AO because of derivatization of the carboxyl groups and steric hindrance). Some conjugates, however, could not be measured with the same accuracy, e.g., for Vi-DT, the linearity of the initial slope deviated up to ±20%. The difficulty encountered in titrating certain conjugates could be due to the facts that the conjugate solvent was 0.2 M NaCl rather than 0.15 M, the concentration of Vi was limiting, and the conjugate had a low Vi-to-protein ratio, and/or to properties of some carrier proteins. The carrier protein of the Vi-DT conjugates was previously treated with Formalin. FTIR analyses of Vi-DT (see above) were also less accurate than those of the other conjugates such as Vi-CT (CT was a native protein). CD spectroscopy has indicated that the conformation of the *N*-succinimidyl 3-(-2-pyridyldithio)propionate derivative of CTB is similar to that of the native CTB (S. C. Szu and A. L. Stone, unpublished data). These carrier proteins were, therefore, probably largely undenatured after the conjugation procedures. It is presently unknown whether these differences in structure of the conjugate affect the immunogenicity of the vaccine.

Negative charges are likely to be involved in the biological action of the bacterial capsule. The reaction of AO with Vi and Hib suggests that other CPS (e.g., from *Neisseria meningitidis* groups A and X and *E. coli* K100) could be investigated by binding of AO (8, 9, 12, 13, 14, 20). Those CPS that resemble Vi in anionic density could be studied by the binding of AO or MB. MB is particularly suited for comparison of polyanionic structures because the frequency shift and hypochromism of this dye are more sensitive to the geometry of the negative groups on a polysaccharide and relatively greater in extent than those of AO (27, 29). The metachromasia of MB, therefore, might distinguish between anionic structures within or among CPS.

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