INFECTION AND IMMUNITY, Feb. 1978, p. 462-469
0019-9567/78/0019-0462$02.00/0

Vol. 19, No. 2
Printed in U.S.A.

Lipid-Free Glycerol Teichoic Acids with Potent Membrane-Binding Activity

HAROLD R. COOPER, FRANK W. CHORPENNING,* AND SAMUEL ROSEN

Department of Microbiology and the College of Dentistry, The Ohio State University, Columbus, Ohio 43210

Received for publication 28 July 1977

Lipid analysis of several glycerol teichoic acid preparations strongly indicated that covalently bound lipid is not required for spontaneous adsorption of glycerol teichoic acid to erythrocyte membranes. Although fatty acids were detected in each of four batches, none were covalently bound. Chloroform-ether-extracted antigens retained potent erythrocyte membrane-binding activity as measured by passive hemagglutination, even though they were shown to contain less than one fatty acid residue per 4,869 teichoic acid chains. Mild ammonolysis abolished erythrocyte-sensitizing activity in passive hemagglutination, but further studies indicated the loss of activity was due to partial destruction of the polyglycerophosphate backbone and not to the removal of esterified lipid. The amount of hydrolyzed antigen required to produce 100% passive hemagglutination inhibition was between 170 and 330 times the amount required to produce the same result using unhydrolyzed glycerol teichoic acid. The average chain length was reduced from 19.1 to 9.7, 7.4, and 5.1 glycerophosphate residues for antigen samples hydrolyzed for 1, 5, and 16 h, respectively.

Recent interest in glycerol teichoic acid (GTA) has been generated from studies showing that certain GTA preparations have detrimental biological effects. For example, injections of GTA have been reported to suppress the sheep cell response in mice (12, 13) and to potentiate immunological injury in diseases such as experimental arthritis (15) and nephritis (B. A. Fiedel and R. W. Jackson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, abstr. M364, p. 126). GTAs are known to spontaneously adsorb to cell membranes of a variety of tissues, including erythrocytes (RBC) (8, 14), and this property has been postulated to play a central role in potentiating the biological effects of the antigen.

The component responsible for adsorption of GTA to RBC was first reported to be alanyl side chain substituents (8). The evidence for this was primarily based on experiments showing that mild hydrolysis of the antigen removed alanyl esters with the concomitant abolishment of sensitizing activity. However, several studies demonstrated that GTA preparations lacking alanyl esters retained potent RBC membrane-binding activity (3, 5, 7, 16). Hewett et al. (7) observed that mild alkaline hydrolysis cleaved ester-linked lipid as well as alanyl esters and suggested that covalently bound lipid was the component responsible for the affinity of teichoic acid for cell membranes. This suggestion has had wide support and has been strengthened by experiments showing that sensitizing activity could be restored by esterification of the hydrolyzed antigen with fatty acids (11, 16). However, there are opposing arguments. Phosphodiester bonds of polyglycerophosphate (PGP) are also susceptible to alkaline hydrolysis (1). Consequently, the procedures used to cleave alanine and lipid, though milder than those normally used to hydrolyze phosphodiester bonds, could result in partial destruction of the PGP backbone of the antigen. Treatment of GTA with trichloroacetic acid has been reported to remove covalently bound fatty acids (19). Therefore, antigens prepared by methods using trichloroacetic acid would not be expected to sensitize RBC if this property is dependent on covalently bound lipid. There is sufficient evidence, however, to doubt that this is the case. Chorpenning and Stamper (3) utilized trichloroacetic acid as described by Decker et al. (5) in the preparation of a bacillary GTA and obtained antigens that not only retained cell membrane-binding activity but also demonstrated enhanced activity on a weight basis when compared with non-trichloroacetic acid-treated preparations. In view of the implied controversy, the present investigation was undertaken to clarify the involvement of lipid in promoting spontaneous adsorption of GTA to RBC membranes.

MATERIALS AND METHODS

Antigen preparation. Several lots of GTA were prepared from whole cells of a *Bacillus* sp., OSU-372

(17), as described by Decker et al. (5). A fourth lot (PF-GTA) was obtained in precisely the same manner, except all reagents and chromatography columns used for its purification were prepared with pyrogen-free, double-distilled demineralized water (DDD H_2O). All glassware was cleaned with acid cleaning solution and thoroughly rinsed with pyrogen-free DDD H_2O before use. A fifth lot of antigen was prepared from purified cell walls of *Streptococcus mutans* BHT, obtained by the method of Cooper et al. (4). The cell wall material was extracted four times with 4 volumes of chloroform-ether (3:1) before antigen preparation to remove noncovalently bound lipid.

Lipid analysis. Assays designed to detect noncovalently bound lipid were made on chloroform-ether extracts of each purified bacillary and streptococcal GTA preparation. Samples were extracted four times by mixing 1 volume of each antigen solution (2 mg/ml of pyrogen-free DDD H_2O) with 4 volumes of chloroform-ether (3:1) and shaking vigorously for 1 min. After phase separation, the organic fractions were harvested, pooled, and evaporated to dryness under a stream of nitrogen gas. Covalently bound lipid determinations were made on lyophilized chloroform-ether-extracted samples of each bacillary or streptococcal GTA.

Each preparation (chloroform-ether extracts and extracted antigens) was saponified by adding 1 N alcoholic KOH (1 mol of KOH per liter of absolute methanol) and heating for 1 h at 100°C. The saponified material was neutralized to a phenophthalein end point with 1 N alcoholic HCl and evaporated to dryness at 60°C under a stream of nitrogen gas. The residues were suspended in pyrogen-free DDD H_2O (acidified to pH 2.0 with 1 N HCl) and extracted four times with equal portions of chloroform-ether to recover fatty acids. The organic fractions were pooled and extracted again with acidified pyrogen-free DDD H_2O (pH 2.0). After the aqueous extract was discarded and the organic fraction was dried under a stream of nitrogen gas at 60°C, 1 N alcoholic HCl was added to each residue, and the potassium salt of each fatty acid was formed by neutralizing with 1 N alcoholic KOH. The samples again were evaporated to dryness as described above. A sample of corn oil (positive control) and 10 fatty acid standards were treated and prepared in precisely the same manner. Fatty acid standards included hexanoic, octanoic, lauric, myristic, palmitoleic, palmitic, oleic, stearic, arachidic, and lignoceric acid.

Lipid analyses were also done on chloroform-ether extracts of two 50-ml samples of DDD H_2O obtained from different containers and on unextracted samples of the PF-GTA preparation. Water samples were taken from the same source as the water used for routine antigen preparation but represented different lots.

All samples and standards were converted to their respective phenacyl ester derivatives, using an alkylating solution which consisted of acetonitrile containing 60 and 6.0 nmol, respectively, of α,p-dibromoacetophenone (Aldrich Chemical Co., Milwaukee, Wis.) and dicyclohexyl-18-crown-6 (Aldrich Chemical Co.) per ml, as described by Durst et al. (6).

Derivatized fatty acids were identified and quantitated by high-pressure liquid chromatography, using a Tracor 970 variable-wavelength detector and a Tracor 990 isochromatographic pump (Tracor, Inc., Austin, Tex.). The apparatus was equipped with a 20-μl injection loop and a μBondapak/C18 reverse-phase column (Waters Associates, Milford, Mass.). The flow rate was 1.7 ml/min, absorbance was set at 254 nm with the sensitivity selector set at 1, and the chart speed was 0.2 inch (ca. 0.51 cm) per min. Fatty acids of short chain length (C_6-C_{12}) were separated using a mobile phase consisting of 90% methanol–10% water, C_{12} to C_{20} acids were chromatographed using 95% methanol–5% water, and fatty acids of C_{20} to C_{24} were resolved using absolute methanol. Fatty acid peaks were identified by injecting known standards along with samples. Amounts of fatty acid were determined from standard curves prepared by plotting peak height against concentration. Experiments with derivatized standards demonstrated the lower limits of sensitivity to be between 0.05 and 0.10 nmol/injection (20 μl).

Endotoxin assays. Endotoxin (lipopolysaccharide) contamination of the various preparations was determined by the *Limulus* amebocyte lysate assay for pyrogen (10). The test was carried out using a Worthington pyrostat reagent kit (Worthington Biochemicals Corp., Freehold, N.J.).

Mild alkaline hydrolysis of purified GTA. Samples of chloroform-ether-extracted antigen were dissolved in pyrogen-free DDD H_2O and subjected to mild alkaline hydrolysis, using the method of Hewett et al. (7). Samples were hydrolyzed for 1, 5, and 16 h, respectively, at room temperature, neutralized with 0.1 N HCl, and lyophilized.

Antigen chain length measurements. The effects of mild alkaline hydrolysis on the antigen were determined in terms of chain length measurements made before and after treatment, using the method of Vaught and Bleiweis (18).

Restoration of RBC-sensitizing activity. A sample of chloroform-ether-extracted antigen was hydrolyzed for 16 h in 14.5% ammonium hydroxide (final concentration) to abolish RBC-sensitizing activity. After hydrolysis, neutralization, and lyophilization, the antigen was dissolved in pyrogen-free DDD H_2O and extracted four times with 4 volumes of chloroform-ether. The aqueous fraction containing lipid-free antigen was harvested after the last extraction and lyophilized in an acid-cleaned vial. A sample of the dried antigen was then suspended in *N,N*-dimethyl formamide, and pyridine was added in accordance with the procedure of Ofek et al. (16), except that no chloronated fatty acid derivative was added. The control contained neither solvent. After treatment, the antigen was tested for RBC-sensitizing activity by passive hemagglutination (PHG) as described below.

Serological tests. Samples of unextracted, chloroform-ether-extracted, and hydrolyzed GTA were assayed for their relative RBC-sensitizing activity by PHG checkerboard titration and for antibody-binding capacity by PHG inhibition. RBC (rabbit) coating and PHG testing were performed as described by Chorpenning and Stamper (3).

PHG inhibition was measured by incubating constant amounts of each GTA preparation with arithmetic dilutions of a standard anti-PGP-antiserum as

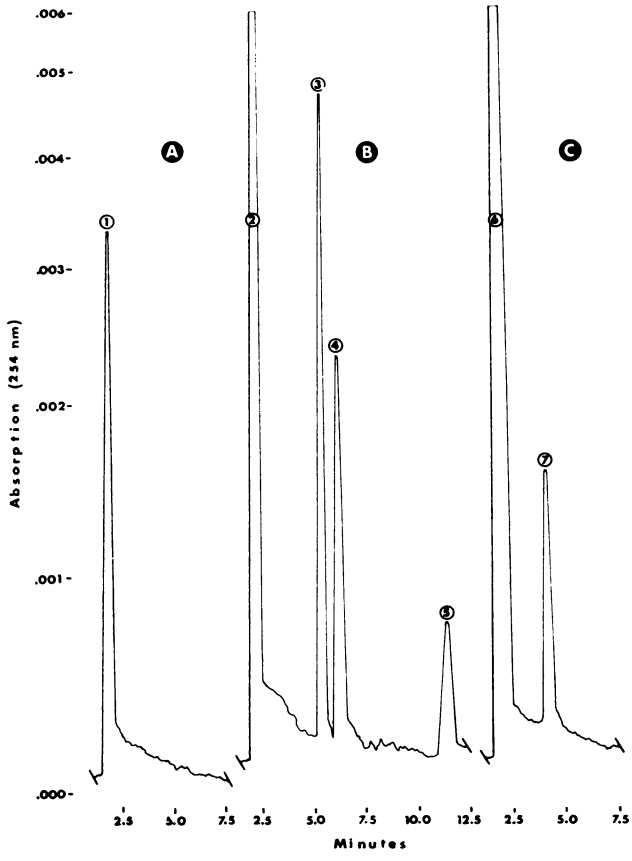

FIG. 1. *Noncovalently bound fatty acid analysis of a 5-μg sample of bacillary GTA. Profiles A, B, and C were obtained using mobile phases of 90% methanol–10% water, 95% methanol–5% water, and absolute methanol, respectively. Peaks were identified, using internal standards, as α,p-dibromoacetophenone (1, 2, and 6), palmitoleic derivative (3), palmitic derivative (4), and arachidic derivative (5 and 7).*

described elsewhere (2). Rabbit RBC suspensions optimally coated with a standard GTA preparation were then added, incubated, and read for PHG inhibition.

RESULTS

High-pressure liquid chromatograms of chloroform-ether extracts (noncovalently bound lipid) from three lots of purified bacillary GTA and one lot of purified *S. mutans* BHT cell walls consistently revealed the presence of several fatty acids in small quantities. A representative profile of the chloroform-ether extracts (5 μg) is presented in Fig. 1, and the quantities of fatty acids detected in all lots are shown in Table 1. In contrast to the noncovalently bound lipid assays, covalently bound fatty acid residues could not be detected in any of the four chloroform-ether-extracted antigens, using samples up to 1.0 mg of GTA per injection (Fig. 2 is a representative profile). The reliability of the fatty acid assay was verified using corn oil samples as positive controls. Analysis of corn oil samples resulted in quantitative recovery of the constituent fatty acids myristic, palmitic, palmitoleic, linoleic, oleic, and stearic.

Since no covalently bound lipid was detected in the purified GTA, and in view of the inconsistency in terms of the noncovalently bound fatty acids present, experiments were done to determine whether DDD H_2O, similar to that used in the preparation of the antigens, contained detectable fatty acid levels. High-pressure liquid chromatograms of hydrolyzed and derivatized chloroform-ether extracts from two different samples of DDD H_2O consistently demonstrated low levels of three or four different fatty acids (Table 2). Additional evidence for lipid contamination from DDD H_2O was provided by the *Limulus* amebocyte lysate assay for endotoxin. Absorbance readings for duplicate samples of DDD H_2O alone and samples containing bacillary GTA were well beyond the recommended range for quantitation. However, by subtracting DDD H_2O absorbance values from those ob-

TABLE 1. *Noncovalently bound fatty acid content of purified GTA from whole cells of Bacillus sp. 372 and cell walls of S. mutans BHT*

GTA lot[a]	Fatty acid	Mean peak ht (mm)	nmol/assay[b]	Mean concn	
				nmol/mg	μg/mg
1	Palmitoleic	158.1	0.94	188	47.8
	Palmitic	94.2	0.72	144	36.9
	Arachidic	32.9	0.44	88	27.5
2	Palmitoleic	140.5	0.84	168	42.7
	Palmitic	16.5	0.13	26	6.7
	Oleic	13.0	0.10	20	5.1
	Stearic	65.0	0.64	128	36.1
	Unidentified[c]	9.6	0.10	20	5.7
3	Lauric	50.0	0.27	54	10.8
	Myristic	7.0	0.04	8	1.8
	Palmitic	35.4	0.27	54	13.8
	Stearic	18.8	0.18	36	10.2
S. mutans	Myristic	12.3	0.07	1	3.2
	Palmitic	25.1	0.19	38	9.7
	Stearic	15.9	0.15	30	8.5

[a] Numbered lots are bacillary extracts.
[b] Determined directly from standard peak height curves using samples representing 5 μg of purified GTA.
[c] Quantitated as palmitic acid.

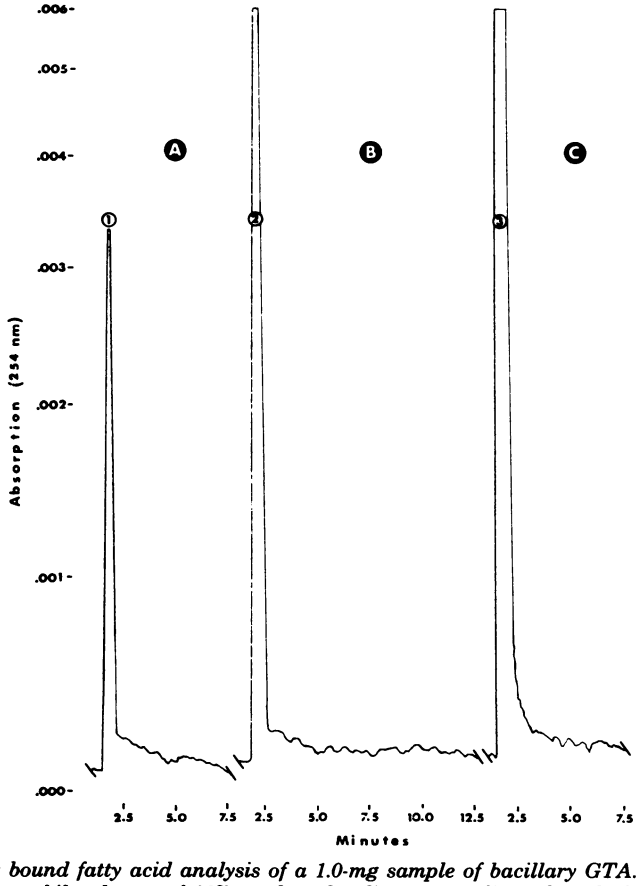

FIG. 2. *Covalently bound fatty acid analysis of a 1.0-mg sample of bacillary GTA. Profiles A, B, and C were obtained using mobile phases of 90% methanol–10% water, 90% methanol–5% water, and absolute methanol, respectively. All three peaks were identified, using internal standards, as α,p-dibromoacetophenone (1, 2, and 3).*

TABLE 2. *Fatty acid content of DDD H_2O samples*

Sample	Fatty acid	Mean peak ht (mm)	Mean concn	
			nmol/ml[a]	ng/ml
1	Myristic	151.2	0.80	182.7
	Palmitic	90.5	0.70	179.5
	Arachidic	99.2	0.39	121.9
2	Palmitic	178.0	1.31	335.9
	Stearic	30.5	0.30	85.3
	Arachidic	12.0	0.16	50.0
	Unidentified[b]	10.0	0.09	23.0

[a] Determined directly from standard peak height curves using samples representing 1.0 ml of DDD H_2O.

[b] Quantitated as palmitic acid.

tained from samples containing antigen, it was found that between 0.4 and 0.8 ng of lipopolysaccharide contaminant was present per milligram of antigen. *Limulus* amebocyte lysate assays of the pooled GTA sample became negative following chloroform-ether extraction.

Since fatty acid and endotoxin analyses indicated that the probable source of lipid in the antigen preparations was DDD H_2O, bacillary GTA prepared with pyrogen-free buffers and reagents was examined for fatty acid content. High-pressure liquid chromatograms of samples representing up to 1.0 mg of hydrolyzed and derivatized pyrogen-free GTA were consistently negative. This assay represented a total fatty acid analysis (covalently and noncovalently bound fatty acids) since the antigen was not extracted with chloroform-ether before hydrolysis. *Limulus* amebocyte lysate assays were also negative using up to 1.0 mg of pyrogen-free GTA per test.

Spontaneous adsorption of both chloroform-ether-extracted and unextracted GTA to RBC membranes was demonstrated with two separate GTA preparations. Results of one such checkerboard titration are shown in Table 3. In both instances, chloroform-ether extraction was found to enhance RBC membrane-binding activity on a weight basis. The amount of unextracted GTA antigen required to optimally coat RBC for PHG was 60 and 50 μg, respectively. However, only 20 and 25 μg was required to produce the same result using chloroform-ether-extracted samples of the same two lots of antigen. In contrast to the enhancement of membrane-binding activity, chloroform-ether extraction was found to have virtually no effect on the antibody-binding capacity of each antigen tested, as measured by PHG inhibition (Fig. 3).

Experiments to determine the effects of mild alkaline hydrolysis on purified GTA preparations showed that hydrolysis completely abolished RBC-coating activity after 1, 5, and 16 h of hydrolysis in 14.5% ammonium hydroxide. In addition to destroying membrane-binding activity, hydrolysis greatly decreased the antibody-binding capacity of the antigen, as measured by PHG inhibition assays (Fig. 4), and significantly reduced the average chain length (Table 4).

Results of subjecting the hydrolyzed GTA to the conditions that Ofek et al. (16) used to esterify palmitic acid to the glycerol residues were interesting. Although samples of up to 1.0 mg failed to coat RBC for PHG after being mildly hydrolyzed for 15 h in 14.5% ammonium hydroxide, significant restoration of RBC-sensitizing activity was achieved by this treatment in the absence of free fatty acids. At the same time, untreated controls remained negative. PHG checkerboard titrations (Table 5) revealed that 1.0 mg of GTA that was hydrolyzed and then treated would optimally coat RBC and quantities as low as 50 μg were sufficient to give a clear 4+ reaction with undiluted antiserum. Although these results indicate a significant degree of restored membrane-binding activity, they do not represent complete restoration of activity.

DISCUSSION

Spontaneous adsorption of GTA to RBC membranes has been reported to be due to covalently bound lipid (7, 11, 16). The purified teichoic acids (streptococcal and bacillary) used in the present study retained their cell membrane-binding property after trichloroacetic acid treatment and ion-exchange chromatography. This retention of PHG activity is significant from two standpoints; first, treatment with 10% trichloroacetic acid cleaves covalently bound lipid (9, 20), and, second, antigens were eluted from a cation-exchange column, which irreversibly binds GTA with covalently bound lipid (9, 20). These facts strongly indicated that the purified GTA preparations did not contain covalently bound lipid. This suggestion was clearly confirmed by lipid analysis. Although fatty acids

TABLE 3. *PHG checkerboard titrations of unextracted and chloroform-ether-extracted bacillary GTA*

Antigen (GTA)	Concn (μg/ml)	Agglutinin titer (standard anti-GTA antiserum)
Unextracted	65	128
	60	128
	55	64
	50	64
Extracted	25	128
	20	128
	15	64
	10	16

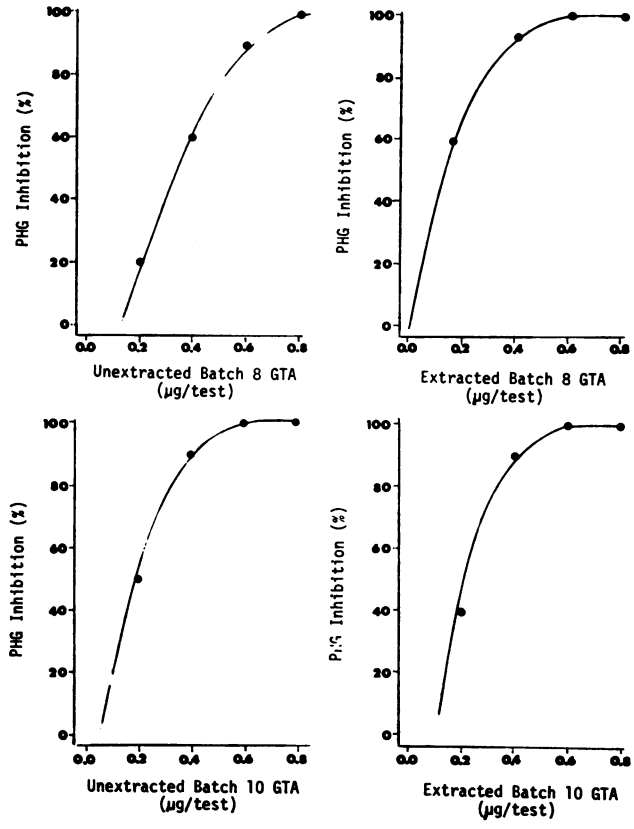

FIG. 3. *Inhibition of PHG by unextracted and chloroform-ether-extracted samples of bacillary GTA. Tests were carried out using rabbit RBC optimally coated with GTA. The antiserum was a standard rabbit anti-GTA antiserum raised against whole cells of Bacillus sp. (OSU-372).*

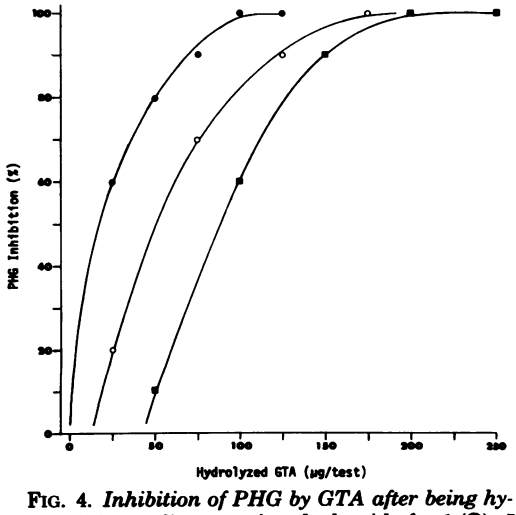

FIG. 4. *Inhibition of PHG by GTA after being hydrolyzed in 14.5% ammonium hydroxide for 1 (●), 5 (○), and 16 (■) h, respectively. The tests were carried out using rabbit RBC optimally coated with GTA. The antiserum was a standard rabbit anti-GTA antiserum raised against Bacillus sp. (OSU-372) whole cells.*

TABLE 4. *Chain length analysis of bacillary GTA after ammonolysis*

Ammonolysis (h)	Phosphorus (μmol/mg)[a]		Mean chain length[b]
	Total[c]	Terminal	
0	4.65	0.24	19.1
1	4.65	0.48	9.7
5	4.65	0.63	7.4
16	4.65	0.91	5.1

[a] Determined directly from a standard phosphorus curve.

[b] Computed as micromoles of total phosphorus/micromoles of terminal HPO_3.

[c] Total phosphorus determinations were done only on the original material.

were detected in each of the four lots of antigen assayed, none were covalently bound since lipid was not detected in samples after chloroform-ether extraction. The detection of both lipid and endotoxin in water samples but not in the antigen (PF-GTA) prepared with pyrogen-free reagents strongly suggests that the lipid component observed in each of the four purified GTA preparations was a contaminant and not a con-

TABLE 5. *PHG checkerboard titration of hydrolyzed GTA which was then treated with N,N-dimethyl formamide and pyridine*

Antigen concn (μg/ml)	Agglutinin titer (standard anti-GTA antiserum)		
	Hydrolyzed-treated	Hydrolyzed-untreated	Non-hydrolyzed
1,000	128	0	NT[a]
750	64	0	NT
500	64	0	NT
250	16	0	64
100	8	0	128
50	8	0	64

[a] NT, Not tested.

stituent of the purified antigen. This suggestion is further supported by the lack of consistency in fatty acids between lots of bacillary antigen.

Some investigators feel that less than 1% lipid by weight is sufficient for the antigen to retain affinity for cell membranes (16). It is difficult to prove total elimination of esterified fatty acids, but in view of the sensitivity of the assay used, chloroform-ether-extracted antigens clearly contain less than 0.05 nmol/mg of sample. This represents a maximum fatty acid-to-glycerol molar ratio of 1:93,000 or approximately 1 fatty acid residue per 4,869 GTA chains (based on a mean chain length of 19.1 glycerophosphate residues). In view of these computations, it is difficult to associate the membrane-binding property of GTA with lipid. In fact, PHG checkerboard titrations of unextracted and chloroform-ether-extracted antigens in conjunction with inhibition studies indicate that the lipid component actually inhibits adsorption of GTA to RBC. This is evident from the observation that the membrane-binding capacity of the antigens was increased threefold by chloroform-ether extraction (Table 3), whereas extraction had no apparent effect on the antibody-binding capacity of the antigens (Fig. 3). The increased membrane-binding activity of the antigens cannot be explained solely in terms of purification, since lipid accounted for less than 4% of each antigen tested.

Evidence supporting the claim that lipid is required for spontaneous adsorption of GTA to RBC membranes is based on experiments showing the abolishment of sensitizing activity by mild ammonolysis (7, 16) and studies demonstrating the restoration of membrane-binding activity after esterification of fatty acid residues onto the PGP gackbone of the hydrolyzed antigen (11, 16). The present study indicates, however, that mild alkaline hydrolysis results in the partial destruction of the antigen. This conclusion is based on the facts that hydrolysis not only destroyed PHG activity but dramatically decreased the PHG-inhibiting capacity of the antigen and significantly reduced the average chain length of the PGP backbone. Consequently, it appears that methods used to remove covalently bound lipid also result in the partial destruction of the PGP backbone. Thus, destruction of the polymer rather than removal of lipid probably accounts for the loss in sensitizing activity. These results collectively appear to indicate that the membrane-binding property of GTA is related to chain length.

The finding that membrane-binding activity could be restored to the hydrolyzed antigen in the total absence of fatty acids or their derivatives may explain why others (11, 16) attributed membrane-binding activity to fatty acid content. Yet, it is difficult to explain this restoration of activity. Ofek et al. (16) reported that RBC-sensitizing activity that had been abolished by mild ammonia hydrolysis could be restored by condensation of palmitoyl chloride with the hydrolysis product. Since we had evidence that fatty acids were not needed for adsorption, we thought it desirable to repeat that procedure in the absence of any fatty acids to determine if such manipulation affected the hydrolyzed material. Restoration of membrane-binding activity occurred. It would be attractive to conclude that restoration of activity was due to the re-esterification of phosphodiester bonds. However, we have no explanation as to how the treatment could do this. We can only state that the hydrolyzed GTA lost its membrane-binding activity upon depolymerization and regained it during exposure to dimethyl formamide and pyridine.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant DE 02719 from the National Institute of Dental Research and by a grant from The Ohio State University Development Fund.

We thank Robert Kates for the use of his high-pressure liquid chromatography apparatus.

LITERATURE CITED

1. **Archibald, A. R., and J. Baddiley.** 1966. The teichoic acids. Adv. Carbohydr. Chem. **21**:323–375.
2. **Chorpenning, F. W., H. R. Cooper, and S. Rosen.** 1975. Cross-reactions of *Streptococcus mutans* due to cell wall teichoic acid. Infect. Immun. **12**:586–591.
3. **Chorpenning, F. W., and H. B. Stamper.** 1973. Spontaneous adsorption of teichoic acid to erythrocytes. Immunochemistry **10**:15–20.
4. **Cooper, H. R., F. W. Chorpenning, and S. Rosen.** 1975. Preparation and chemical composition of the cell walls of *Streptococcus mutans* BHT. Infect. Immun. **11**:823–828.
5. **Decker, G. P., F. W. Chorpenning, and G. T. Frederick.** 1972. Naturally-occurring antibodies to bacillary teichoic acids. J. Immunol. **108**:214–222.
6. **Durst, H. D., M. Milano, E. J. Kikta, Jr., S. A. Connelly, and E. Grushka.** 1975. Phenacyl esters of fatty

acids via crown ether catalysts for enhanced ultraviolet detection in liquid chromatography. Anal. Chem. 47:1797-1801.

7. **Hewett, M. J., K. W. Knox, and A. J. Wicken.** 1970. Studies on the group F antigen of lactobacilli: detection of antibody by haemagglutination. J. Gen. Microbiol. 60:315-322.
8. **Jackson, R. W., and M. Moskowitz.** 1966. Nature of a red cell-sensitizing substance from streptococci. J. Bacteriol. 91:2205-2209.
9. **Knox, K. W., and A. J. Wicken.** 1973. Immunological properties of teichoic acid. Bacteriol. Rev. 37:215-257.
10. **Levin, J., and F. Bang.** 1964. The role of endotoxin in the extracellular coagulation of *Limulus* blood. Bull. Johns Hopkins Hosp. 115:265-274.
11. **Matsuno, T., and H. D. Slade.** 1971. Group A streptococcal polysaccharide antigens. Infect. Immun. 3:385-389.
12. **Miller, G. A., and R. W. Jackson.** 1973. The effects of a *Streptococcus pyogenes* teichoic acid on the immune response of mice. J. Immunol. 110:148-156.
13. **Miller, G. A., J. Urban, and R. W. Jackson.** 1976. Effects of a streptococcal lipoteichoic acid in host responses in mice. Infect. Immun. 13:1408-1417.
14. **Moskowitz, M.** 1966. Separation and properties of a red cell-sensitizing substance from streptococci. J. Bacteriol. 91:2200-2204.
15. **Ne'eman, N., and I. Ginsburg.** 1972. Cell sensitizing antigen of group A streptococci. II. Immunological and immunopathological properties. Isr. J. Med. Sci. 8:1807-1816.
16. **Ofek, I., E. H. Beachey, W. Jefferson, and G. L. Campbell.** 1975. Cell membrane binding properties of group A *Streptococcal lipoteichoic* acid. J. Exp. Med. 141:990-1003.
17. **Stamper, H. B., and F. W. Chorpenning.** 1975. An experimentally pathogenic *Bacillus sp.* I. Description and characterization of the organism. Can. J. Microbiol. 21:485-489.
18. **Vaught, R. M., and A. S. Bleiweis.** 1974. Antigens of *Streptococcus mutans*. II. Characterization of an antigen resembling glycerol teichoic acid in walls of strain BHT. Infect. Immun. 9:60-67.
19. **Wicken, A. J., and K. W. Knox.** 1970. Studies on the group F antigen of lactobacilli: isolation of a teichoic acid-lipid complex from *Lactobacillus fermenti*. J. Gen. Microbiol. 60:293-301.
20. **Wicken, A. J., and K. W. Knox.** 1975. Lipoteichoic acids: a new class of bacterial antigen. Science 187:1161-1167.