THE OCCURRENCE OF POLYGLYCEROPHOSPHATE AS AN ANTIGENIC COMPONENT OF VARIOUS GRAM-POSITIVE BACTERIAL SPECIES

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The present report deals with the identification of a new bacterial substance which was first recognized as a result of its reactivity with certain streptococcal antisera. Extracts of Groups A to G streptococci, as well as those of various other Gram-positive bacteria, give strong precipitin reactions with these antisera, indicating that the substance is widely distributed in the microbial world. Purification and characterization of the active component was carried out principally with material extracted from Group A streptococci, using the serological reaction as a guide during the fractionation procedures. On the basis of the evidence presented below, it is concluded that the active substance is an organic polyphosphate which appears to be a simple polymer built up solely of glycerophosphate units.

Materials and Methods

All the strains of streptococci and some of the staphylococci used in this study were from the lyophilized collection of strains maintained in this department. Representative strains of several other species of bacteria were supplied by Dr. A. W. Bernheimer and Dr. W. Lane Barksdale of New York University College of Medicine, New York. Several lots of frozen, packed Group A streptococcal cells were supplied by Difco Laboratories, Detroit, through the courtesy of Mr. A. L. Lane.

The antisera employed were selected from a large group obtained in the course of preparing typing sera for Group A streptococci. Qualitative serologic tests were carried out by the capillary precipitin method, and quantitative precipitin analyses by the procedure used in studies of the cell wall carbohydrates (1).

The method of Allen (2) was used in the determination of phosphorus and the method of Koch and McMeekin (3) in the determination of nitrogen.

EXPERIMENTAL

Characteristics of the Serological Reaction.—The rabbit antiserum (R54) responsible for the initial recognition of the new bacterial substance was prepared by the repeated intravenous injection of a heat-killed vaccine of a strain of Group A, Type 38 streptococcus (C141/98/1) by the method employed in the production of streptococcal typing antisera. This antiserum, even after adsorption with heterologous Group A cells, reacted strongly with the stock extracts

of Groups A to G streptococci, the stock M extracts of all types of Group A streptococci, and with a preparation of Group A carbohydrate extracted by the hot formamide method of Fuller. However, purified preparations of Group A carbohydrate and of other streptococcal antigens—e.g. Type 1 M and T proteins and Type 28 R antigen—failed to react with the adsorbed serum. Characteristically, the reaction was prompt and vigorous, giving rise to a heavy, flocculating precipitate comparable to that encountered in strong anti-carbohydrate precipitin reactions.

Several additional antisera which formed precipitates with the same materials that reacted with antiserum R54 were subsequently encountered among the antisera prepared with vaccines of other types of Group A streptococci. However, a large number of rabbits are immunized by this general technique for experimental purposes and for the maintenance of the collection of grouping and typing antisera in this laboratory, and only a very small per cent of the total tested were reactive. Thus, it must be concluded that only an occasional rabbit responds with the formation of significant amounts of precipitating antibody to the bacterial component in question. The alternative possibility was considered that the serological reaction is due to the occurrence of an unusual non-antibody substance comparable to C-reactive protein, but this appeared unlikely in view of the pattern of appearance and disappearance of the reactive material during immunization and the demonstration of a clear-cut booster response upon reimmunization of one animal. Furthermore, fractionation of serum R54 by starch electrophoresis in pH 8 veronal buffer revealed that the precipitable substance was sharply limited to the slowest moving (γ_1) portion of the gamma globulin. It appears, therefore, that the formation of true precipitins is involved. Accordingly, the bacterial component is antigenic as it occurs in the intact bacterial cell, and for purposes of convenience the soluble product after extraction from the cell will also be referred to as an antigen even though it may possibly not induce the formation of antibodies in this state.

Extraction and Cellular Localization of the Bacterial Antigen.—It was apparent from the occurrence of the antigen in stock grouping extracts that it is readily released from the cells by boiling at pH 2. Not all of the material is extracted by a single 20 minute treatment of heavy cell suspensions under these conditions, and several extractions are required to bring all of the antigen into solution. The antigen forms an insoluble complex with other constituents of the extract at pH 2–3, and consequently the heated suspension must be neutralized before removing the cells by centrifugation. The stability of the antigen is indicated by its resistance to this treatment and by its retention of serological activity after the even more drastic conditions of extraction with formamide at 160°–180°C. A portion of the antigen may be brought into solution with milder treatments. For example, the use of trypsin or simple extraction of cells at pH 9 and room temperature yields active extracts, although neither of these procedures provides an efficient means for quantitative extraction.

MACLYN McCARTYDisruption of streptococcal cells in the Mickle disintegrator results in quantitative release of the antigen into solution. The cell wall fraction, obtained by differential centrifugation of the suspension of disintegrated cells, contains none of the antigen and none is released by enzymatic dissolution of the walls. It is apparent, therefore, that the antigen differs in this respect from the group-specific carbohydrate and such surface protein antigens as the M, T, and R antigens which occur exclusively in the cell wall fraction. Similarly, the antigen is not found in the finely particulate material which is separated from the disintegrated suspension by relatively high-speed centrifugation. The water-clear supernatant fraction following high-speed centrifugation contains all the antigen, together with the nucleic acids, much of the protein and various other cellular constituents. In the case of *Staphylococcus aureus*, a similar distribution is encountered after disintegration of the cells, and none of the antigen occurs in the cell wall or fine particle fractions. The information on localization of the antigen within the cell is thus chiefly negative in character and indicates that it is not a cell wall component.

Distribution of the Antigen among Other Bacterial Species.—The occurrence of the antigen in many different groups of streptococci suggested that it might have an even wider distribution and be found in unrelated bacterial species. To test this possibility, extracts were prepared of representative strains of a number of different groups of bacteria. For the most part the organisms were grown in an appropriate fluid medium and recovered by centrifugation. As a routine procedure, extraction was carried out by the method used for preparing grouping extracts of Group A streptococci; *i.e.*, boiling a heavy suspension of cells at pH 2. In some cases, however, other methods of extraction were also used, especially in those cases in which unexpected negative results were obtained. Thus, extracts of pneumococci were made by autolysis or rapid lysis initiated with desoxycholate; lysozyme was used with *Micrococcus lysodeikticus*; the *Streptomyces albus* enzymes with some of the antigen-negative streptococci; and Mickle disintegration was employed with various strains. In no case was the antigen obtained by these alternative methods from strains which were negative by the acid extraction procedure. In each case, the extracts were tested with antiserum by the capillary precipitin method.

The results of the survey are presented in Table I. One of the most striking findings is the absence of the antigen in all of the Gram-negative organisms tested. Gram-positive organisms are divided into two large groups with relatively little evidence of variability among different strains of a single species. Prominent among the organisms which contain the antigen are the streptococci, including many of the non-hemolytic species and those that are unclassified serologically, the staphylococci and the aerobic sporulating bacilli. On the other hand, pneumococci, clostridia, corynebacteria, and yeasts appear to lack the antigen consistently. In addition, none was found in the nine strains of Group O streptococci examined. Similarly, most Group H streptococci do not

contain the antigen. Among a large group of non-hemolytic and *viridans* strep-
tococci which had been isolated from the blood of patients with subacute
bacterial endocarditis both positive and negative strains were encountered.

TABLE I

Distribution of Precipitating Antigen in Various Bacterial Species

A. GRAM-POSITIVE ORGANISMS CONTAINING THE ANTIGEN
    Hemolytic streptococcus
        Group A—all types
        Groups B, C, D, E, F, G, K, L, and N
    Streptococcus salivarius
        Types I (4), II (1), and NT (2)*
    Streptococcus MG (1)
    Streptococcus viridans—some strains (14/25)
    Staphylococcus
        aureus (8) and *albus* (3)
    Bacillus
        subtilis (3), *cereus* (1), *anthracis* (2), *megatherium* (1)
    Leuconostoc mesenteroides (1)

B. GRAM-POSITIVE ORGANISMS LACKING THE ANTIGEN
    Pneumococcus (11)
    Streptococcus
        Groups H (5/7) and O (9)
    Streptococcus viridans—some strains (11/25)
    Staphylococcus citreus (2)
    Corynebacterium
        diphtheriae (5), *xerosis* (1), *pyogenes* (5), *ulcerans* (1), *ovis* (1)
    Micrococcus lysodeikticus (2)
    Clostridium
        histolyticum (1), *septicum* (6), *tetani* (1), *sporogenes* (1)
    Yeasts
        Candida (3), *Saccharomyces* (2)

C. GRAM-NEGATIVE ORGANISMS LACKING THE ANTIGEN
    Escherichia coli (2)
    Aerobacter aerogenes (2)
    Proteus morgani (1)
    Salmonella typhi (1) and *paratyphi* (1)
    Neisseria pharynges (1)
    Hemophilus influenzae (1)
    Vibrio metchnikovii (1)

* The numbers in parentheses indicate the number of different strains tested in each case

These streptococci were not identifiable by available serological techniques and
no other distinguishing features were recognized which differentiated the
strains lacking the antigen from those which contained it. The amount of anti-
gen present in the various positive strains appeared to be roughly comparable
in general, although among the endocarditis strains of streptococci definite and

consistent quantitative variations were encountered and some strains possessed only small amounts.

Purification of the Antigen.—Purification of the antigen was facilitated by determination of some of its properties in crude extracts. On zone electrophoresis the antigen migrated rapidly at about the same rate as the ribonucleic acid in the extracts. However, removal of a large part of the ribonucleic acid resulted in an increase—rather than a decrease—of the ratio of phosphorus to specific serological activity of the extract. It was thus strongly suggested that the active substance represented a polyphosphate, and purification schemes were based on this finding.

Purified preparations with comparable properties and chemical analyses were obtained from acid extracts and from the material released by Mickle disintegration of streptococci. Although probably less desirable because of the drastic conditions employed, the acid extraction procedure proved superior from the point of view of reproducibility and yield of final product. This appeared to be referable to the greater complexity of the crude Mickle preparations which contained essentially all of the cellular constituents exclusive of the cell wall, and considerable difficulty was encountered in removing all the protein in the course of purification.

The procedures employed in isolation of the antigens from acid extracts include: (*a*) precipitation of inert material at pH 4, (*b*) alcohol precipitation, (*c*) fractionation of barium salts with alcohol, (*d*) treatment with ribonuclease, (*e*) deproteinization with chloroform, (*f*) treatment with cation exchange resin, and (*g*) final removal of nucleotides on charcoal. The details of the method are exemplified by the following preparation:—

Packed cells of Group A, Type 3 streptococci (D58, Richards) received from Difco Laboratories (1 kilo wet weight) were thawed in 1.8 liters of 0.2 N HCl. The pH of the suspension was brought to 2 with concentrated HCl and the suspension was then heated to 95°C. and held at this temperature with continuous stirring for 20 minutes. After cooling to room temperature and neutralization with 5 N NaOH, the cells were recovered by centrifugation, resuspended and reextracted by the same procedure. The combined extracts were brought to pH 4.1 with 6 N HCl with the formation of a voluminous precipitate. The precipitate, which contained none of the serological activity, was removed by suction filtration and washed with 10^{-4} N HCl. The filtrate and washings were concentrated to about 1 liter by vacuum distillation.

The concentrated filtrate was mixed with an equal volume of absolute ethanol and the resulting precipitate discarded. An additional volume of alcohol was added to the supernate to precipitate the antigen which was removed by filtration and taken up in water. Barium acetate at a final concentration of 2 per cent was added to the solution and the inactive precipitate removed. Further inactive material was obtained by the addition of 0.2 volume of ethanol to the supernate, and the antigen was then precipitated as the barium salt by bringing the alcohol concentration to 50 per cent (*v/v*). After resolution of the precipitate in 100 ml. water, 10 mg. crystalline ribonuclease was added and the mixture incubated for 18 hours at 37°C. The ribonuclease digest was dialyzed against 3 changes of distilled water and subsequently treated twice with chloroform by the Sevag method for deproteinization.

After chloroform treatment, sodium chloride was added at a final concentration of 1 per

cent and the active material precipitated by the addition of 2 volumes of ethanol. The pre-
cipitate was redissolved in water and the yellow solution passed 2 times through a bed of
Dowex 50 X8 (200 to 400 mesh) in the hydrogen form. At this point the material was es-
sentially colorless but contained more than 10 per cent ribonucleotides which were removed
by the use of charcoal. The solution, after neutralization with NaOH, was passed through
a column (2 x 20 cm.) of equal parts of darco G-60 and celite No. 503. The antigen was re-
tained on the column and eluted with ethanol solutions. Elution began with 15 per cent
ethanol and was completed with 25 per cent ethanol. The antigen was precipitated from the
combined eluates by the addition of excess alcohol, washed with absolute ethanol and ether
and dried *in vacuo*. The yield of the sodium salt was 400 mg.

Some variability has been encountered in the behavior of the material on
charcoal columns. On some occasions only part of the serologically active ma-
terial has been retained when the aqueous solution is initially passed through
the column despite careful control of conditions (pH, ratio of charcoal to
amount of material). However, the removal of nucleotides has been effectively
achieved on each occasion regardless of this variability.

Recovery of comparable material from extracts prepared by disintegration
of cells in the Mickle disintegrator has proved more difficult, and the losses
have been much larger. To a large extent, this difference appears to be due to
the presence of large amounts of protein in these extracts which becomes de-
natured in the course of the preparative procedures and holds much of the
antigen in an insoluble combination. Removal of this protein by chemical or
enzymatic means was not completely successful. However, a sufficient quantity
of purified material was obtained by this technique so that the properties
could be compared with the preparations obtained by the more drastic method
of heating at pH 2.

Properties of the Purified Antigen.—By the procedure outlined in the pre-
ceding section, the sodium salt of the antigen is obtained as a dry white powder
which is readily soluble in water. The barium salt has also been prepared and
dissolves in water with somewhat more difficulty. Attempts to dry the free
acid form of the antigen yielded translucent gums. The antigen appears to be
at the border-line of dialyzability through cellophane tubing (Visking), and in
the case of the material extracted by Mickle disintegration less than 1 per cent
passes through the membrane in 24 hours at neutral pH. On the other hand,
the antigen extracted by heating at pH 2 is dialyzable to the extent of 20 to 30
per cent under the same conditions, indicating that some degradation results
during the extraction procedure. There is no detectable serological difference
between the dialyzable and non-dialyzable portions.

Proteolytic enzymes and pancreatic ribonuclease and desoxyribonuclease
have no effect on the antigen. Similarly, phosphatases cause no reduction in
serological activity and only minute amounts of inorganic phosphate are re-
leased. Acid hydrolysis (2 N HCl) rapidly destroys serological activity with the
release of no more than traces of inorganic phosphate. Even 24 hour hydrolysis

at higher acid concentrations splits off only 10 to 20 per cent of the organically bound phosphate. However, in contradistinction to the intact antigen, acid hydrolysates are highly susceptible to alkaline phosphatase, and the total phosphorus is quantitatively converted to inorganic phosphate by action of the enzyme.

Qualitative Chemical Analysis.—The purified antigen is essentially nitrogen-free, and the absence of nucleotides is confirmed by the fact that solutions show little or no adsorption between 250 and 300 $m\mu$. The high phosphorus content directed attention to this component in attempts to determine the composition of the material. Paper chromatography of acid hydrolysates showed no evidence of carbohydrate or amino acids, but with the solvent systems described by Hanes and Isherwood (4) for use with phosphate esters the presence of a single organic phosphate was revealed. The mobility of the organic phosphate was identical with that of glycerophosphate. With one of the solvent systems (tertiary amyl alcohol:formic acid:water) three organic phosphate spots were obtained, but sodium-α-glycerophosphate behaved similarly when subjected to the same conditions of hydrolysis. With a solvent system (tertiary butanol:picric acid:water) in which α and β glycerophosphates show significant differences in mobility, the mobility of the organic phosphate in hydrolysates of the antigen was identical with that of α-glycerophosphate. There was little evidence of material with the mobility of β-glycerophosphate or of possible glycerodiphosphates.

Further evidence for the occurrence of glycerophosphate as the predominant unit of the antigen was obtained by analysis of the dephosphorylated product. Acid hydrolysates of the antigen were treated with alkaline phosphatase and compared with phosphatase-treated glycerophosphate and with known polyols by the paper chromatographic method of Hough (5), using water-saturated butanol as solvent. A single polyol spot was obtained, identical in its mobility with glycerol and the dephosphorylated glycerophosphate.

Elementary Chemical Analysis.—Completely anhydrous preparations of the antigen were not obtained for analysis. As in the case of the sodium glycero-phosphates, high vacuum drying over P_2O_5 at room temperature removes only part of the associated water, and even treatment at temperatures up to 90°C. caused only a gradual further loss of water. There is a possibility also that some of the organic solvent (*e.g.* ethanol) used in precipitating the antigen may be bound, since certain samples of the sodium salt had high C:P ratios despite the fact that glycerol was the only polyol demonstrable on analysis.

The data recorded in Table II represent analytical data on the barium salts of two preparations of the antigen. The differences from the theoretical values for the barium salt of polyglycerophosphate are accounted for only in part by the presence of water. However, the ratio of C:P in preparation IV, which was made by the more favorable acid-extraction procedure, is close to the theoretical

value of 3.0. The remainder of the discrepancy can be accounted for by the high ash values, presumably dependent upon the incorporation of excess barium in conversion to the barium salt.

Serological Properties of Purified Antigen.—The purified antigen shows the same precipitating behavior with antisera as the crude extracts, giving rise to

TABLE II

Elementary Analysis of Antigen

	C	H	P	Ash
	per cent	*per cent*	*per cent*	*per cent*
Prep. IV—Ba salt...........................	12.99	3.31	10.96	65.6
Prep. M—from Mickle supernate—Ba salt.......	14.68	3.45	11.26	63.5
Theory for $(C_3H_6O_5PBa_{1/2})_n$...................	16.24	2.72	13.97	

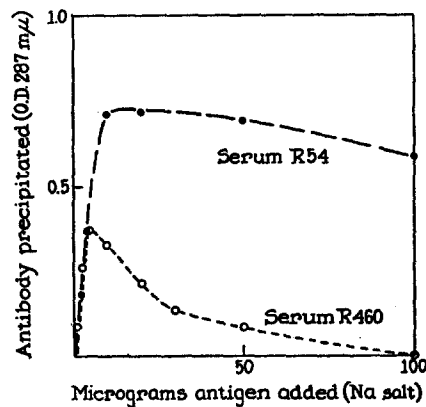The elementary analysis was carried out by Mr. Theodore Bella.

Fig. 1. Quantitative precipitin curves with purified antigen and streptococcal antisera Serum R54 is the original serum which called attention to the occurrence of the antigen.

heavy flocculent precipitates which appear almost immediately after mixture of antigen and antiserum. Despite the probable degradation of the acid-extracted material, it gives quantitative precipitin curves identical with those of the material obtained from Mickle disintegrated cells. Quantitative curves with representative antisera are illustrated in Fig. 1. The curve with R54, the original antiserum which called attention to the occurrence of the antigen, is somewhat unusual in that only a minimal inhibitory effect is found in the region of excess antigen. Among the several antisera studied, R54 is unique because of its high potency and rapidity of reaction, and the available evidence indicates that the lack of inhibition in moderate antigen excess reflects the rapidity with which

insoluble complexes are formed and their resistance to resolution rather than heterogeneity of the antigen-antibody system. The precipitin curve with R460 is typical of the other antisera which react with the antigen, and shows a more characteristic sharp zone of maximum precipitation followed by marked inhibition in the region of antigen excess. Antisera of this type have been useful in specific inhibition studies designed to confirm the chemical nature of the antigen.

Specific Inhibition Studies.—Sodium α- and β-glycerophosphates cause some nhibition of the precipitin reaction with the antigen, but only at relatively
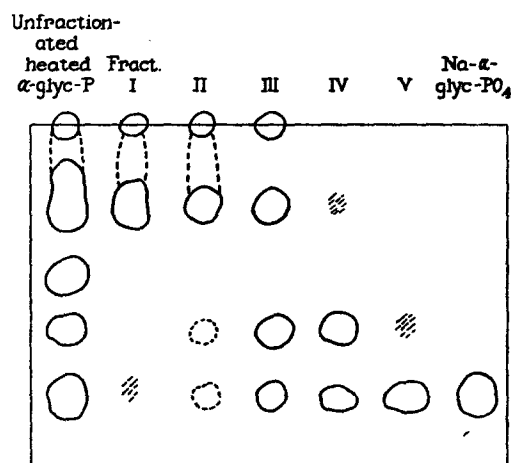

FIG. 2. Paper chromatographic pattern of product obtained by treating α-glycerophosphoric acid in the anhydrous state and of fractions of this material.

high concentrations. On the basis of experience with other antigen-antibody systems, it seemed probable that small polymers of glycerophosphate would be much more effective as inhibitors. Accordingly, an attempt was made to prepare mixed polymers by the condensation of anhydrous glycerophosphoric acids.

For this purpose, sodium α-glycerophosphate was converted to the free acid by passing a 10 per cent solution over a bed of Dowex 50 in the hydrogen form. The solution was dried over P_2O_5 under vacuum, and the thick oily residue subjected to 105°C. under varying conditions. The material was then neutralized with NaOH.

Material obtained in this way consistently showed 10 to 20 times the inhibitory activity of untreated glycerophosphate. It represented a heterogeneous mixture as illustrated by the complex pattern observed on paper chromatography (Fig. 2). Clean fractionation of this mixture was not achieved, but by application of the paper chromatographic technique on a semi-preparative scale

partial separation was possible. The pattern of the fractions obtained in this way are illustrated in Fig. 2. Fraction I is composed chiefly of the slower moving components, fraction III of the intermediate components, and fraction V mostly of material with a mobility like that of untreated glycerophosphate. These fractions were tested for inhibitory activity using antiserum R460 and purified antigen at the equivalence point.' The results are recorded in Fig. 3,

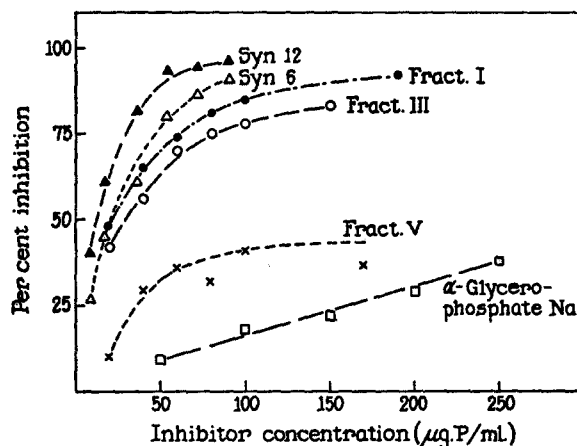FIG. 3. Specific inhibitory effect of fractions of heated α-glycerophosphate and of synthetic polyglycerophosphate. Reaction system: Serum R460 + bacterial polyglycerophosphate at equivalence point + inhibitors at various concentrations on basis of phosphorus content. Fract. I, III, and V are those illustrated in Fig. 2. Syn 6 and syn 12 represent synthetic calcium polyglycerophosphates with average chain length of 6 and 12 units, respectively.

which gives the percentage inhibition observed at various concentrations (on the basis of phosphorus content) of each fraction.

The data in Fig. 3 show that fraction I, which contains the slower moving material and thus presumably the larger complexes, has the highest inhibitory activity. Fraction III has less activity and fraction V the least, although it is somewhat more effective than α-glycerophosphate. None of the fractions have any inhibitory effect on other antigen-antibody systems; e.g. Group A carbohydrate and its antibody. Even though the identity of the heterogeneous material is not defined, the fact that fraction I causes 50 per cent inhibition at such low concentrations (20 μg. P/ml.) supports the view that the antigen is a polyglycerophosphate. More definitive evidence was subsequently obtained by the use of synthetic polyglycerophosphates prepared by Michelson by a more appropriate chemical method. The findings with this material are described in the next section.

Studies with Synthetic Polyglycerophosphate.—Two samples of the calcium

salt of synthetic polyglycerophosphate were generously supplied by Dr. Michel-son.[1] The product obtained by his method is heterogeneous in molecular size (6), and the average chain length of the two samples used in our studies was approximately 12 and 6 units respectively. In all probability, therefore, the average size of the synthetic polymers is considerably smaller than that of the natural polyglycerophosphate obtained from streptococci.

Serological tests showed that both preparations of synthetic polyglycero-phosphate react to form precipitates with serum R54. The results of qualitative precipitin tests are given in Table III. As would be expected, the material with an average chain length of 12 units gives better and more rapidly developing

TABLE III

Qualitative Precipitation Tests with Synthetic Polyglycerophosphate and Serum R54

Initial antigen concentration	Antigen		
	Syn-12	Syn-6	Bacterial polyglycerophosphate
mg./ml.			
1.0	++±	+±	+++
0.2	+±	±	++++
0.04	±	0	++++
0.01	0	0	++
0	−	−	0

Capillary precipitin tests, ++++ = maximal precipitation; 0 = no precipitation.

Syn-12 and Syn-6 = calcium salts of synthetic polyglycerophosphate with average chain length of 12 and 6 respectively.

reactions than the 6 unit material. In both cases considerably higher concentrations are required for the formation of visible precipitation than in the case of the bacterial antigen. This suggests that only the small fraction of long chain material in the heterogeneous mixture is capable of reacting, and that the special properties of antiserum R54 are responsible for the apparent lack of inhibition by the larger amount of short chain material present. The behavior of the synthetic polyglycerophosphate in other antisera (*e.g.* R460) is in accord with this view. In this case, the synthetic material does not form precipitates at any concentration but strongly inhibits the reaction with the bacterial anti-gen. Quantitative data on this inhibitory effect with serum R460 are included in Fig. 3. It is apparent that these synthetic polyglycerophosphates are the most potent inhibitors among the materials studied and that inhibition is greatest with the preparation of longer average chain length.

The remaining supply of serum R54 was not sufficient for detailed quantitative precipitin analysis with the synthetic polyglycerophosphates. However, a few tests carried out in a concentration range suggested by the qualitative tests indicate that the 12-unit material can precipitate approximately one-half as much antibody as the bacterial antigen (Table IV). It can be predicted that synthetic polyglycerophosphate of longer chain length (20 or more units) would behave like the natural bacterial product in serological testing.

Infrared spectroscopy was employed in the course of attempts to identify the bacterial antigen.[2] The spectrum of the purified material showed some resemblance to the spectra of simple α- and β-glycerophosphates, but differences

TABLE IV

Quantitative Precipitins with Synthetic Polyglycerophosphate and Serum R54

Antigen	Amount antigen added	Antibody N precipitated per 0.1 ml. serum
	mg.	*µg. N*
Syn-12	0.1	29
"	0.2	32
Purified streptocococcal polyglycerophosphate	0.005	54
" " "	0.01	60

Reaction system, 0.1 ml. serum in final volume of 1.0 ml.

Syn 12, calcium salt of synthetic polyglycerophosphate with average chain length of 12.

were present which could only tentatively be attributed to polymerization. More satisfactory information was obtained by comparison of the spectra of the natural bacterial product and Michelson's synthetic polyglycerophosphate. This comparison is shown in Fig. 4, and it is evident that the two substances are for the most part spectroscopically identical. Minor differences may be in part attributable to the greater water content of the bacterial material and to the different cations present. The results when taken together with the chemical and serological evidence, leave little doubt concerning the identity of the bacterial antigen.

Estimation of Size of the Bacterial Polyglycerophosphate.—If one assumes that the natural polyglycerophosphate is an unbranched chain with a single terminal phosphate, an estimation of the average chain length can be made by the use of phosphatase. The results of an experiment of this type, in which the antigen was treated for 20 hours with alkaline intestinal phosphatase (Armour), are illustrated in Table V. The activity of the enzyme was controlled by measuring

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[2] Infrared spectroscopy of the various products was made possible by the generous cooperation of Dr. Herbert Jaffe.
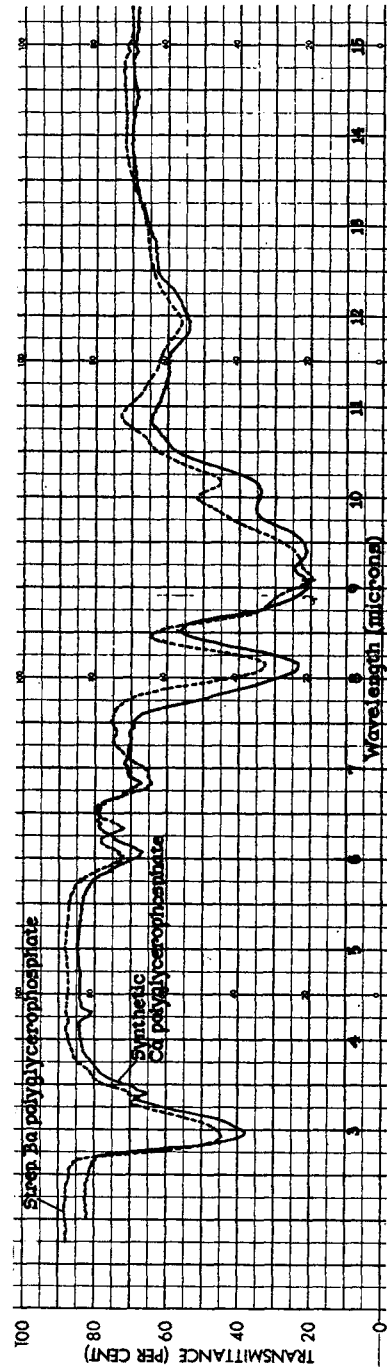
FIG. 4. Comparison of infrared spectra of synthetic polyglycerophosphate (calcium salt, average chain length of 6 units) and streptococcal polyglycerophosphate (barium salt, prep. IV). Samples incorporated in KBr pellets.

its effect on sodium α-glycerophosphate. Phosphatase treatment removed 1.78 per cent of the total phosphorus from the polyglycerophosphate. This indicates a chain length of 56 units or a molecular weight of approximately 10,000 for the sodium salt. The material employed in this analysis had been prepared by dis-

TABLE V

Estimation of Average Chain Length of Bacterial Polyglycerophosphate

Substrate	Analysis after Phosphatase Treatment*	
	Total P	Inorganic P
	μg.	*μg.*
Streptococcal polyglycerophosphate, Ba salt 23.9 mg.....	2470	44
Sodium α-glycerophosphate, 10 mg. (enzyme control)....	970	980

* Alkaline intestinal phosphatase (Armour) for 20 hours at pH 9 and 37°C.

TABLE VI

Immunochemical Estimation of Polyglycerophosphate Content of Streptococci

Antigen	Amount of antigen added	Antibody precipitated O.D. at 287 mμ	
		Without inhibitor	With inhibitor
	μg.		
Purified streptococcal polyglycerophosphate	0.5	0.038	0.003
	1.0	0.065	0
	1.5	0.120	0
	2.0	0.139	0
	3.0	0.180	0.002
	ml.		
Crude extract of Group A streptococci (Supernate of	0.01	0.045	0
Mickle disintegrated cells)*	0.02	0.119	0.012
	0.03	0.170	0.021
	0.04	0.202	0.030

System: 0.2 ml. antiserum—final volume 1 m

* Total volume of crude extract = 75 ml. from 654 mg. streptococcal cells (dry weight).

integration of streptococci rather than acid-heat extraction and was essentially non-dialyzable through standard cellophane tubing.

Quantity of Polyglycerophosphate in the Bacterial Cell.—The losses during isolation of purified polyglycerophosphate from bacteria are too large and irregular to allow an accurate estimate of the total amount present in the cells. In order to obtain information on this point, quantitative immunochemical procedures were used in measuring the amount of polyglycerophosphate in unfractionated bacterial extracts. The possibility of other antigenic substances

in the crude extract contributing to the precipitate was controlled by a parallel series of tests using specific inhibitor (material comparable to fraction I in Fig. 3) at a concentration which completely eliminates the reaction with poly-glycerophosphate. An example of the procedure used is as follows:—

654 mg. of acetone-dried Group A streptococci were suspended in water and disintegrated with glass beads in the Mickle disintegrator. The total volume after thorough washing of the beads was 75 ml. The suspension was clarified by high speed centrifugation, and quantitative precipitin analysis was carried out on the supernate with antiserum R460 in comparison with purified preparations of streptococcal polyglycerophosphate.

The results are recorded in Table VI. Under the conditions of this experiment, the inhibitor effectively eliminated precipitin formation with the purified antigen. On the other hand, the crude extract gave small but appreciable amounts of precipitate in the presence of inhibitor, presumably due to the occurrence of other, unidentified antigens in the extract. After correcting for this non-specific precipitate, the data indicate that the crude extract contains the equivalent of 70 μg./ml. of purified sodium polyglycerophosphate. The total amount is thus 5.25 mg. or 0.8 per cent of the dry weight of the streptococcal cells. Similar tests with other strains of streptococcus and with staphylococci gave comparable values with a range of 0.8 to 1.2 per cent.

DISCUSSION

The present investigation indicates that a polymer of glycerophosphate is present in appreciable amounts in various Gram-positive bacteria, and that it can induce antibody formation, at least in the form in which it occurs in Group A streptococci. That the presence of polyglycerophosphate was not encountered earlier in the course of the extensive serological work with streptococci is probably explained by the fact that under ordinary conditions antibody to this component is removed in the process of adsorption of antisera to render them type-specific. The production of significant amounts of antibody to this antigen is obviously not a common event, but once an unusually potent antiserum had called attention to its occurrence a number of other unadsorbed sera were shown to have some degree of reactivity with the antigen.

The occurrence of glycerophosphate as a significant component of certain bacterial species was demonstrated in chemical studies by Mitchell and Moyle (7). Their investigations were initiated by the discovery that lipide-free extracts of staphylococci contained a significant amount of phosphorus over and above that which could be accounted for by the nucleic acid content. The component principally responsible for this "excess phosphate" was identified as glycerophosphate, although smaller amounts of an unidentified polyol phosphate were present. The material analyzed by Mitchell and Moyle was obtained by alkaline extraction and contained glycerophosphate in the simple, non-polymerized

form, but these workers suggested that the substance might occur as a polymer in the intact organism.

The results of a survey of various microbial species by Mitchell and Moyle (8) for the occurrence of "excess phosphate" by their technique closely parallels those obtained in the serological test for polyglycerophosphate reported in the present study. The chief discrepancy is in the case of yeasts and *Corynebacterium xerosis* which they found to contain appreciable amounts of "excess phosphate" but which appear to lack the serologically active polyglycerophosphate. Mitchell and Moyle noted a certain parallelism between the occurrence of "excess phosphate" and the Gram-positive staining reaction, although they recognized that the clostridia provided a notable exception. They did not test pneumococci and certain streptococci, such as those of Group O, which may well provide additional exceptions since they lack the antigenic polyglycerophosphate.

In attempting to determine the localization of glycerophosphate in the staphylococcal cell, Mitchell and Moyle (9) obtained results which are completely at variance with those reported in the present paper. They found the great bulk of the "excess phosphate" to be present in the cell wall fraction after disruption of staphylococci in the Mickle disintegrator and concluded that a glycerophosphate-protein complex is the major constituent of the staphylococcal cell wall. On the other hand, in the present investigation none of the serologically active polyglycerophosphate was found in the cell wall fraction of Group A streptococci or the strains of staphylococci studied. However, the amount of polyglycerophosphate estimated to be present in the cells by our immunochemical procedure is roughly comparable to the amount of "excess phosphate" which Mitchell and Moyle found in the soluble fraction. It would appear that the antigenic substance accounts for only a portion of the non-nucleotide, non-lipide phosphorus of these bacteria.

The recent studies of Baddiley and his colleagues (summarized in reference 10) have thrown considerable light on the mechanism of bacterial synthesis of polyglycerophosphate. On the basis of their isolation and identification of cytidine diphosphate ribitol and cytidine diphosphate glycerol from *Lactobacillus arabinosus*, they postulated the occurrence of ribitol phosphate and glycerophosphate polymers. Subsequently they isolated a ribitol phosphate polymer, designated as teichoic acid, and demonstrated that it is present in large amounts in the cell walls of *Lactobacillus arabinosus*, *Bacillus subtilis*, and *Staphylococcus aureus*. The presence of a glycerophosphate polymer was also established, but in this case, in agreement with the studies reported in the present paper, it was definitely not a component of the cell wall. While Baddiley and his group have not studied the glycerophosphate compound in as much detail as the teichoic acid, there appears to be little doubt that it is the same as the serologically active substance described above.

In view of the foregoing, teichoic acid must have been a component of the "excess phosphate" encountered by Mitchell and Moyle, and possibly ribitol phosphate represents the unidentified polyol phosphate which they found in staphylococci. The occurrence of this substance may account in part for the different conclusions concerning the localization of polyglycerophosphate in the cell reached by Mitchell and Moyle on one hand and Baddiley and ourselves on the other.

The possible significance of polyglycerophosphate in the bacterial cell remains obscure. Studies are in progress on the cellular localization of the antigen, and the results may conceivably provide a clue to its function.

SUMMARY

A bacterial substance has been described which gives a precipitin reaction with certain antisera to Group A streptococci. The precipitating antigen is present in various Gram-positive bacteria, including most hemolytic streptococci, staphylococci, and aerobic sporulating bacilli. It is not present in any of the Gram-negative species examined or in pneumococci, clostridia, or corynebacteria.

Analysis of purified preparations obtained from Group A streptococci indicates that the antigen is a simple polymer of glycerophosphate. The identification has been confirmed by immunochemical studies, including precipitin tests and specific inhibition with synthetic polyglycerophosphates. In addition, the infrared spectra of bacterial and synthetic polyglycerophosphate are shown to be closely similar. Immunochemical analysis suggests that the amount of polyglycerophosphate present in Group A streptococci and staphylococci is approximately 1 per cent of the dry weight of the cells. The cellular localization and function of the polyglycerophosphate have not been established.

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