Immunization of Humans with Polyribophosphate, the Capsular Antigen of *Hemophilus influenzae*, Type b

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ABSTRACT In human volunteers, single injections of purified polyribophosphate elicited antibodies detectable by passive hemagglutination and by serum bactericidal and opsonizing activities against viable *Hemophilus influenzae*, type b. All three activities rose by 2 wk to maximal levels, at which they remained for at least 6 months. Doses of 1 μg elicited antibody responses in nearly all recipients; higher doses of the antigen, however, produced larger increases in titer. Booster doses of 1 μg given at 6 months did not further increase the antibody titers. A tuberculin-like response was often observed at the site of injections given intradermally.

INTRODUCTION

Immunization of humans with purified capsular polysaccharides of the pneumococcus has long been known to protect against nasopharyngeal carriage of and systemic infection by homologous strains (1). Similar results have recently been reported with the capsular polysaccharide isolated from a group C meningococcus (2). Evidence cited or reported in an accompanying communication (3) indicates that human antibodies to polyribophosphate (PRP),[1] the capsular antigen of *H. influenzae*, type b, may protect against infections by this bacterium. Humans can acquire these antibodies by asymptomatic (4) or clinical (5) infection with the organism, and anti-PRP antibodies can be induced in animals by immunization with bacterial cultures, if encapsulation is intact (6). Attempts to elicit antibodies in either humans or animals with the isolated capsular antigen, however, have not been reported. The present paper describes the results of immunization of human adult volunteers with purified PRP.

METHODS

Polyribophosphate. PRP, sodium salt, was isolated from *H. influenzae* b, strain Eagan (3), by a modification[2] of the method of Zamenhof and Leidy (7). Chemical assays on the preparation (Table I) were done as follows: pentose, by the orcinol method using D-ribose as the standard (8); phosphate, by the Fiske-Subbarow method (9); sodium, by flame photometry with a lithium internal standard (model 143; Instrumentation Laboratory Inc., Watertown, Mass.); protein, by the Lowry, Rosebrough, Farr, and Randall method (10) with bovine albumin as standard; and nucleic acid (roughly), by optical density at 260 nm, assuming $E^{1\,cm}_{1\%} = 250$. An equimolar ratio of pentose, organic phosphate, and sodium was found, corresponding to the description of PRP by Zamenhof and colleagues (11, 12). These three moieties account for two-thirds of the weight obtained by drying a sample of PRP solution to constant weight at 100°C; since residual water may be trapped in the resulting film, their proportion of the actual dry weight may be closer to unity. Because of the difficulty in controlling moisture content, the PRP concentration of solutions was quantitated by pentose assay. Assuming a formula weight of 225 for one sodium phosphoribosyl unit (11), 1 μg of pentose was taken to equal 1.5 μg of the polymer.

The pentose and phosphorus of the preparation was completely precipitable by rabbit antiserum to strain Eagan. In Ouchterlony gel diffusion the preparation formed a single precipitin line, which was identical with the major of two lines formed by a sonic extract of strain Eagan. Electrophoresis in gel followed by precipitation with antiserum produced a single arc (Fig. 1).

This work was presented in part at the 62nd Annual Meeting of the American Society for Clinical Investigation, Inc., Atlantic City, N. J., May 1970.

Miss Wetterlow is Senior Bacteriologist at the State Laboratory Institute of the Massachusetts Department of Public Health. Dr. Smith is the recipient of Career Development Award AI-20376 of the National Institute of Allergy and Infectious Disease.

Received for publication 21 May 1971 and in revised form 19 July 1971.

[1] *Abbreviations used in this paper:* BC, bactericidal; PBS, phosphate-buffered saline; PHA, passive hemagglutination; PRP, polyribophosphate.

[2] Anderson, P., and D. H. Smith. Manuscript in preparation.

TABLE I

Chemical Assays on the Polyribophosphate Preparation

Assay	% weight*	moles/mole pentose
Pentose	44	(1)
Phosphorus		
Inorganic	0	
Organic	10	1.11
Sodium	7.4	1.07
Nucleic acid	0.83	
Protein	0.36	

* % of that weight determined by drying a water solution of PRP to constant weight at 100°C.

When analyzed by gel filtration chromatography on Sephadex G-200 (Pharmacia Fine Chemicals Inc., Piscataway, N. J.), $\geqslant$ 99% of the PRP pentose and phosphorus eluted in the void volume. For dextrans, exclusion from this gel denotes a molecular weight of $\geqslant 10^5$ (13). In gel filtration on Sepharose 4B (Pharmacia Fine Chemicals Inc.) the bulk of the PRP eluted as a single sharp peak with slight tailings of larger and smaller components (Fig. 2). The small leading "peak" is due to concentration of the higher molecular weight tail in the void volume.) The distribution coefficient, K_{av}, of the apex was 0.4. For dextrans, this value corresponds to a molecular weight of 4×10^5 (13).

PRP for injection was dissolved in phosphate-buffered saline (PBS) (3) containing 0.01% thiomersal, filter sterilized (Millipore Corp., Bedford, Mass.), dispensed into glass injection vials, and stored at 4°C. These operations did not reduce precipitability with antiserum or change the gel filtration profile from that shown in Fig. 2. The specifications of the Division of Biologics Standards were used in testing the injectable preparation for sterility and animal toxicity. In the latter, mice receiving 100 μg and guinea pigs receiving 500 μg intraperitoneally were found to gain weight normally. When pyrogen-tested intravenously in rabbits, the PRP solution was nonpyrogenic at 0.5 μg/kg (near the maximum human dose); injections of 50 μg/kg and 750 μg/kg produced slight fever, but no other observable effect.

Population immunized. Recipients of PRP were volunteers from the groups of adults whose preimmunization titers in type b bactericidal and passive hemagglutination assays had been determined (3). Initially, only those with relatively low titers were given PRP, and intradermal injection was employed for clear observation of local responses. After some experience, volunteers with relatively high titers and the subcutaneous route were included in the study.

Antibody assays. The collection and storage of sera, the antibody assays, and the test strain *H. influenzae* b-Eagan have been described (3). Multiple serum samples from the same individual were tested simultaneously.

Experimental infection of rats with H. influenzae b. To prepare bacteria for experimental infection chocolate agar slants were inoculated, and after 5 hr of incubation the cells were suspended in 0.15 M NaCl. The suspension was chilled in ice water, and its optical density was determined. The bacteria were immediately diluted in 0.15 M NaCl to 10-fold the desired concentration and then diluted 1:10 in a diluent consisting of 0.15 M NaCl plus 5% hog gastric mucin (Wilson Laboratories, Chicago, Ill.) with the pH adjusted to 7 with NaOH after autoclaving. This suspension was held at 0°C and used within 1 hr. Sprague-Dawley rats (COBS-CD; Charles River Breeding Labs, Inc., Wilmington, Mass.) were obtained as weanlings (about 21 days old) and challenged 1 day after delivery to the new quarters. The rats received 0.5 ml of the bacterial suspension intraperitoneally, and survival was scored twice daily.

RESULTS

Kinetics of antibody response. Three initial recipients were injected intradermally with 10, 50, or 55 μg PRP, and frequent serum samples were taken for passive hemagglutination (PHA) assay and serum bactericidal (BC) and opsonization assays against *H. influenzae* strain b-Eagan (Fig. 3). In all three recipients PHA and BC activity increased sharply at about 1 wk, ceased rising by 2 wk, and remained stable for at least 16 wk.

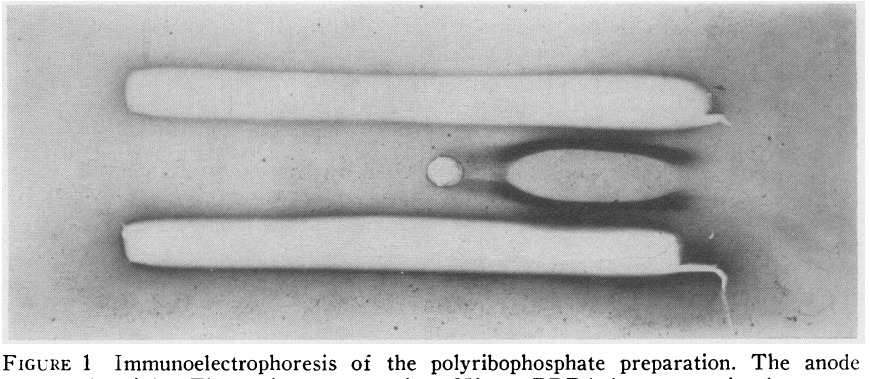

FIGURE 1 Immunoelectrophoresis of the polyribophosphate preparation. The anode was to the right. The antigen preparation, 250 μg PRP/ml, was put in the center well. After electrophoresis 30 min at 30 ma and at room temperature, the troughs were filled with antisera to *H. influenzae,* type b: *lower,* to strain Eagan; *upper,* to strain 62B. After overnight incubation and thorough washing, the slide was stained with Ponceau S.

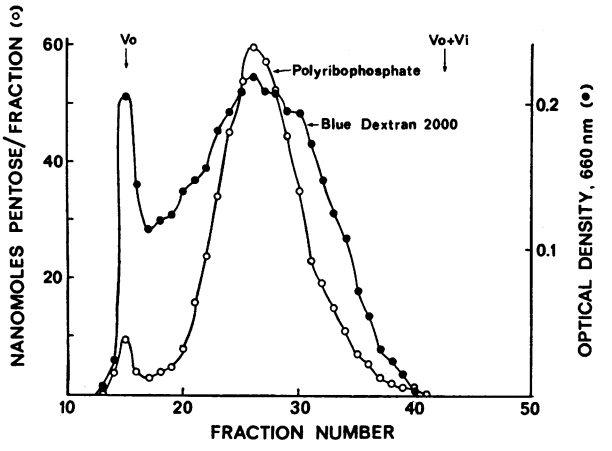

FIGURE 2 Gel filtration profile of polyribophosphate on Sepharose 4B. The column, 2.3 cm diameter, was packed with 203 cm^3 of gel at a hydrostatic pressure of 50 cm and equilibrated with PBS containing 0.02% NaN_3. Samples were eluted by ascending flow at 4.9 ml/hr. Calibration was with 18 mg of Blue Dextran 2000 ($\overline{\text{mol wt}} = 2 \times 10^6$; Pharmacia Fine Chemicals Inc.) applied in 1 ml PBS. The sample was 250 μg PRP in 1 ml PBS.

Although a slight increase in opsonization was detectable as early as 4 days, this activity likewise reached a stable plateau by 2 wk. The kinetics of the responses were the same in recipient B.L., who had detectable preimmunization activity in the (PRP-specific) PHA assay, as in the two with PHA titers of < 2.

To explore the effect of dose on the persistence of the response, the PHA and BC titers at 2–3 wk and at 24 wk were compared in the above three plus five additional recipients of doses varying from 1 to 55 μg. In PHA activity all eight had responded sharply by 2–3 wk and maintained identical titers through 24 wk, with the possible exception of a one dilution drop in two recipients immunized with 10 and 55 μg. In seven of the eight individuals the BC titers had increased sharply by 2–3 wk, and in all seven the 24 wk titer was identical with that at 2–3 wk. One recipient of 55 μg (preimmunization titer of 16) showed no increase at either time point.

The effect of dose and route of administration on the response. A total of 62 adult volunteers received PRP in doses of 0.01–100 μg, intradermally or subcutaneously. Their responses in the BC and PHA assays at 2–3 wk are summarized in Table II. Doses of 0.01 μg did not stimulate antibody production in the two recipients tested; 0.1 μg produced significant increases in roughly half, and 1 μg or greater, in practically all recipients.

The numbers and design are not adequate for a definitive consideration of the relative amounts of antibody generated; however, a relation of dose to antibody increase was apparent: among only those individuals with significant increases (last column) intradermal doses of 1 μg gave greater rises than 0.1 μg (for BC, $P < 0.001$; for PHA, $P = 0.072$) but less than 50 μg (for BC, $P < 0.001$; for PHA, $P = 0.006$). A similar relationship was found with subcutaneous injections. Intradermal and subcutaneous injections were equally effective in producing significant rises. The former route, however, seemed to generate larger increases at the higher doses. Significant mean differences between the two routes were found in PHA at 1 μg ($P = 0.016$) and at 10 μg ($P = 0.046$). Intradermal doses of 50 μg produced greater rises than 100 μg subcutaneously in both BC ($P = 0.002$) and PHA ($P = 0.036$).

Not evident in the Table is the finding that at doses of 1 μg or less failure to respond was coordinate between the two assays, i.e., an individual not responding in BC did not respond in PHA. This was not true for the occasional failures above 1 μg: one recipient had a substantial rise in BC but not in PHA activity, and four had rises in PHA but not BC. In general, individuals with detectable preimmunization PHA activity responded about the same as those without. The four recipients of $\geqslant 1$ μg who had no rise in BC activity all had relatively high titers before immunization.

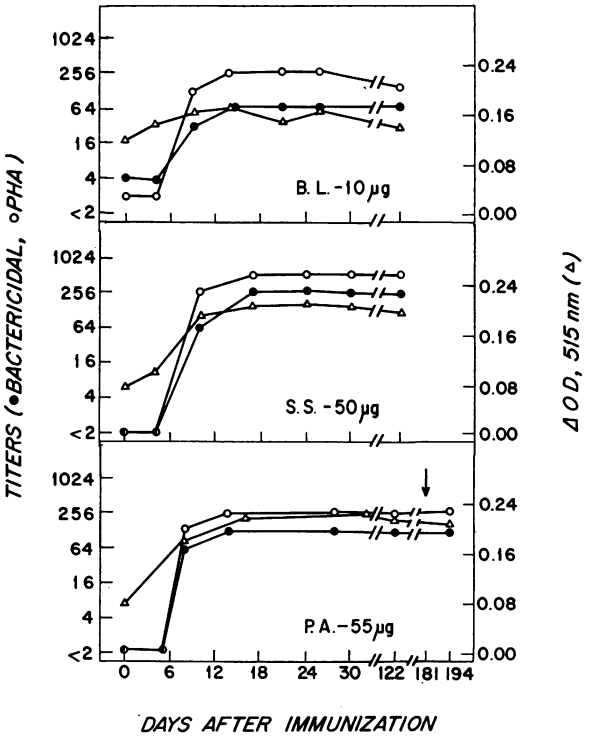

FIGURE 3 The kinetics of response in passive hemagglutinating, bactericidal, and opsonizing activities after immunization with PRP. The arrow indicates the time at which recipient P. A. was given a "booster" injection of 1 μg. Opsonization activity is given in the unit Δ OD, 515 nm (3).

TABLE II

The Effect of Various Doses of PRP Given Intradermally or Subcutaneously on the Bactericidal and Passive Hemagglutination Activities of Adult Volunteers

Dose	Route	Activity	Fraction with increase*	Geometric mean of reciprocal titers‡		Mean rise in those with an increase§	
				Preimm.	Postimm.	Twofold dilutions	SE
μg							
0.01	ID	BC	0/2	22	22	—	—
		PHA	0/2	5.6	8.0	—	—
0.1	ID	BC	3/7	6.6	13	2.0	±0.47
		PHA	3/7	3.6	11	3.7	±0.73
0.1	SC	BC	4/7	7.3	16	1.8	±0.22
		PHA	6/7	3.0	22	3.5	±0.51
1	ID	BC	8/10	4.0	53	4.6	±0.56
		PHA	9/10	2.0	56	5.3	±0.55
1	SC	BC	5/6	2.5	14	3.0	±0.72
		PHA	5/6	2.8	25	3.8	±0.33
3	ID	BC	3/3	5.0	81	4.0	±0
		PHA	3/3	2.5	200	6.3	±1.0
10	ID	BC	8/9	5.4	50	3.6	±0.47
		PHA	9/9	1.4	69	5.7	±0.56
	SC	BC	7/7	4.9	35	2.9	±0.74
		PHA	7/7	3.3	58	4.1	±0.52
50 or 55	ID	BC	3/4	2.4	91	7.0	±0
		PHA	4/4	1.2	180	7.3	±0.40
100	SC	BC	5/7	7.2	64	4.4	±0.81
		PHA	6/7	2.0	58	5.7	±0.64

ID, intradermal; SC, subcutaneous; BC, bactericidal activity against strain b-Eagan; PHA, passive hemagglutination.

* The number of individuals with an increase greater than one twofold dilution/the number injected.

‡ The antilog of the mean of the logs of the reciprocal titers. For this calculation titers of <2 were assumed to be 1 and titers of ⩾256 were assumed to be 256.

§ The mean of the difference in twofold dilutions, calculated for only those with increases greater than one dilution.

Effect of a second injection. Five recipients who had responded to intradermal doses of 3–55 μg were given a second injection, 1 μg intradermally, at 6 months after the initial immunization. No change in antibody activity was detectable in any of the five. One of these was given an additional 1 μg booster 10 months after the first and again did not respond.

Side reactions. In general, injection of the PRP preparation was innocuous. About three-fifths of those receiving intradermal injections had a delayed erythema accompanied, in a few instances, with induration at the site of the injection. The response usually became maximal after 1 day, disappeared by 2 days, and was tender but not painful. The occurrence of the response appeared to be unrelated to dose or to previous antibody titers, but where it did occur, its size was dose related: in several individuals receiving injections of 5 and 50 μg in opposite forearms, the maximal diameter of erythema averaged 35 and 45 mm, respectively. No visable reactions were produced by the subcutaneous injections; four of seven recipients of the 100 μg doses reported a slight tenderness. Two individuals, both recipients of 10 μg intradermally, reported systemic symptoms: one had headache, malaise, and fatigue the following day, and the other had a mild frontal headache lasting about a week. Causative factors other than the injections, however, could not be excluded. One of the five recipients of a 1 μg booster dose had an immediate wheal and flare response, which attained a maximal diameter of 25 mm at ½ hr and disappeared within 3 hr. The individual manifesting this reaction had 60 μg of precipitable anti-PRP antibody/ml of serum (PHA titer of 320) at the time of reimmunization.

Passive immunization with anti-PRP serum. The activity of the human antibody elicited by PRP was further explored through passive protection in experimental infection of weanling rats (Table III). Postimmunization serum, 0.4 ml, given intravenously 1 hr before infec-

tion protected the animals against roughly 10 times the LD_{50} dose of *H. influenzae* b given intraperitoneally; 0.4 ml of preimmunization serum of the same individual had no protective effect.

Immunization of experimental animals. Preliminary attempts to demonstrate an antibody response to PRP in laboratory animals have been negative. Mice, rats, and rabbits given a wide range of doses by several routes and schedules never developed serum PHA activity. The rabbits failing to respond to PRP had not been rendered tolerant, for when subsequently immunized with formalin-killed *H. influenzae* b, they responded with the normal high titers of PHA activity.

DISCUSSION

In normal adults as little as 0.1 μg of PRP elicited significant increases in antibody titer. Higher doses, however, generated somewhat greater increases. Thus, per unit weight, PRP appears to be comparably potent with pneumococcal (14) or meningococcal (15) polysaccharides as a human immunogen. Antibody activities to PRP remained at constant, maximal levels for at least 6 months. Such kinetics are characteristic of other bacterial polysaccharides and have been suggested to result from persistence of these antigens in the immune system (14).

In general, serum bactericidal and opsonizing activities against *H. influenzae* b rose in parallel with activity in the PRP-specific PHA assay. There were, however, four recipients in which a relatively high bactericidal titer did not rise along with an increase in PHA. A possible explanation is offered by the finding that bactericidal activity of anti-PRP antibodies can be masked by bactericidal antibodies directed against somatic antigens (3).

Of the 53 recipients with antibody rises, 30 had had prior experience with the antigen as evidenced by detectable PHA activity, while 23 had preimmunization titers of <2. The relative increase in the two groups, however, was not significantly different. It seems likely that the latter group also had had previous experience with the antigen but that their circulating antibody had dropped below the detectable limit. The possibility is not excluded, however, that this was their first immunizing contact with the antigen and thus that initial and subsequent responses to PRP can be similar. Booster injections of 1 μg given at 6 months, when antibody titers were still maximal, did not increase the titers. This result may have a trivial explanation—a higher dose or different route of administering the antigen might have worked. The reason for the failure, however, might be fundamental. Second injections of pneumococcal polysaccharides do not further increase antibody titers in humans (14). Mice immunized with SIII (16) and rabbits immunized with Type III pneumococci (17) do not respond to a booster dose of SIII with a true secondary response—the antibody level seldom exceeds that from the primary immunization. Siskind, Paul, and Benacerraf have suggested that pneumococcal polysaccharide, though able to stimulate sensitized cells to produce antibody, may be relatively inactive in causing the multiplication of antibody-producing cells (16).

TABLE III

The Effect of PRP Immunization on the Passive Protection by Human Serum of Weanling Rats against Experimental Infection with H. Influenzae b-Eagan

Serum injected (i.v.)	Bacteria injected (i.p. 1 hr after serum)	Survival* at 24 hr	Survival* at 96 hr
None	1×10^5	4/4	2/4
None	1×10^6	3/4	2/4
None	1×10^7	2/6	0/6
0.4 ml preimm.‡	1×10^7	1/5	0/5
0.4 ml postimm.§	1×10^7	5/6	5/6

* Animals surviving/animals injected.
‡ Normal serum of P.A.-titers: BC < 2, PHA < 2.
§ Serum of P. A. 4 months after immunization with 55 μg PRP; titers: BC 160, PHA 320.

Immediate dermal wheal and flare reactions had been observed after the intradermal inoculation of PRP in some patients given therapeutic doses of rabbit anti-*H. influenzae b* serum intravenously (18). For this reason the initial immunization studies were performed primarily with low doses of antigen in individuals with low PHA titers. An immediate wheal and flare was observed only at the reimmunization of one individual, who at the time had a high serum concentration of anti-PRP antibody. A tuberculin-like reaction, however, was manifested by many individuals immunized intradermally. Unlike the reactions provoked by vaccines containing endotoxin, it was delayed, was not accompanied by local pain or systemic symptoms, and was not observed in individuals immunized subcutaneously. Conceivably, this delayed response to PRP is cell mediated. It will therefore be of interest in further studies to observe the reaction to PRP of individuals with deficient cellular immunity and infants who have not had contact with *H. influenzae* b, and to investigate the mitogenic activity of PRP in lymphoid cells *in vitro*.

The experimental infection of rats, in which anti-PRP human serum was protective, is a promising model for study of the pathogenesis of *H. influenzae*. Animals succumbing are found to have a massive bacteremia, and the virulence of various strains is well correlated with their status of encapsulation.

H. influenzae type b is the most common cause of bacterial meningitis in infants and young children (19) and

the cause of a number of other quite serious pediatric infections. The observations that PRP can elicit a long-lived antibody response without serious adverse reactions and that the antibodies are bactericidal and opsonic in vitro and protective in laboratory animals supports the thesis that immunization of humans with PRP might confer active immunity to *H. influenza* b. The importance to the public health of *H. influenzae* b disease encourages further studies of the potential of PRP as a human immunogen.

ACKNOWLEDGMENTS

We are grateful to Hei Sun Lee for technical assistance and to Dr. George Segal for performing the flame photometric assay.

This investigation was supported by National Institutes of Health Grants AI-05877 and 1-FO3-AI 46905-01 from the National Institute of Allergy and Infectious Disease and grants from the Milton Fund, the Medical Foundation, Inc., the Hood Foundation, and the Maternal and Child Health and Crippled Children's Services Program of the Massachusetts Department of Public Health.

REFERENCES

1. MacLeod, C. M., R. G. Hodges, M. Heidelberger, and W. G. Bernhard. 1945. Prevention of pneumococcal pneumonia by immunization with specific capsular polysaccharides. *J. Exp. Med.* **82**: 445.
2. Artenstein, M. S., R. Gold, J. G. Zimmerly, F. A. Wyle, H. Schneider, and C. Harkins. 1970. Prevention of meningococcal disease by group C polysaccharide vaccine. *N. Engl. J. Med.* **282**: 417.
3. Anderson, P., R. B. Johnston, Jr., and D. H. Smith. 1972. Human serum activities against *Hemophilus influenzae,* type b. *J. Clin. Invest.* **51**: 31.
4. Turk, D. C., and C. A. Green. 1961. Measurement of antibodies reacting with capsular antigens of Hemophilus influenzae. *J. Clin. Pathol. (London).* **17**: 294.
5. Peter, G., S. Greenfield, V. M. Howie, and D. H. Smith. 1971. Acquisition of type-specific antibodies following exposure to *Hemophilus influenzae* meningitis. Abstract of the Society for Pediatric Research 41st Annual Meeting. 76.
6. Alexander, H. E., and M. Heidelberger. 1940. Agglutinin and precipitin content of antisera to *Hemophilus influenzae,* type b. *J. Exp. Med.* **71**: 1.
7. Zamenhof, S., and G. Leidy. 1954. Further studies on poly-ribophosphate and other poly-sugarphosphates. *Fed. Proc.* **13**: 327.
8. Ashwell, G. 1957. Colorimeteric analysis of sugars. *Methods Enzymol.* **3**: 73.
9. Leloir, L. F., and C. E. Cardini. 1957. Acid lability of phosphorus compounds. *Methods Enzymol.* **3**: 843.
10. Lowry, O. H., N. J. Rosebrough, L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265.
11. Zamenhof, S., G. Leidy, P. L. Fitzgerald, H. E. Alexander, and E. Chargaff. 1953. Polyribophosphate, the type-specific substance of Hemophilus influenzae, type b. *J. Biol. Chem.* **203**: 695.
12. Rosenberg, E., and S. Zamenhof. 1961. Further studies on polyribophosphate. *J. Biol. Chem.* **236**: 2845.
13. *In* Beaded Sepharose 2B-4B-6B. 1969. Pharmacia Fine Chemicals Inc., Piscataway, N. J.
14. Heidelberger, M., C. M. MacLeod, S. J. Kaiser, and B. Robinson. 1946. Antibody formation in volunteers following injection of pneumococci or their type-specific polysaccharides. *J. Exp. Med.* **82**: 303.
15. Artenstein, M. S., R. Gold, J. G. Zimmerly, F. A. Wyle, W. C. Branche, Jr., and C. Harkins. 1970. Cutaneous reactions and antibody response to meningococcal group C polysaccharide vaccines in man. *J. Infect. Dis.* **121**: 372.
16. Siskind, G. W., W. E. Paul, and B. Benacerraf. 1967. The use of radioactively labeled SIII to study the blood clearance and the immune response of mice to pneumococcal polysaccharides. *Immunochemistry.* **4**: 455.
17. Paul, W. E., G. W. Siskind, B. Benacerraf, and Z. Ovary. 1967. Secondary antibody responses in haptenic systems: cell population selection by antigen. *J. Immunol.* **99**: 760.
18. Dingle, J. H., and L. R. Seidman. 1941. Specific polysaccharide as cutaneous test for evaluation of serum therapy in influenza bacillus meningitis. *Proc. Soc. Exp. Biol. Med.* **46**: 34.
19. Sell, S. H. W. 1970. The clinical importance of *Hemophilus influenzae* infections in children. *Pediat. Clin. N. Amer.* **17**: 415.