The locus of action of embryo juice or mesenchyme in either epidermis or pancreatic epithelium is undefined. Operationally, presence of these factors results in compact, nonspreading epithelia with higher mitotic rates than are seen in the spreading explants incubated in their absence. In the case of epidermis, the active factor could be described as antispreading, orientative, or promitotic, but until the mechanism of action is elucidated, none of the terms is particularly useful. Furthermore, since cytodifferentiation of outer epidermal layers or inner pancreatic acini[10] is apparently dependent upon earlier mesenchyme or embryo juice-induced mitosis, it is not clear how the factors relate to first-order differentiative changes in maturing cells. Utilization of nonliving substrates, defined nutrients, and better-defined particulate fractions lends hope for more penetrating analyses of mitosis, tissue mass, and differentiation in the future.

Summary.—Basal cells of embryonic epidermis remain oriented and mitotically active if cultured *in vitro* on a suitable substrate and in the presence of chicken embryo juice or a macromolecular fraction therefrom. Millipore filters, tropocollagen gels, or frozen-thawed dermis are effective substrates, whereas lens paper or 100,000-*g* centrifugal pellets are not. The active fraction from embryo juice is nondialyzable, heat-labile, trypsin-sensitive, and sediments between 2000 and 100,000 *g*.

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*THE ROLE OF D-ALANINE IN THE SEROLOGICAL SPECIFICITY OF GROUP A STREPTOCOCCAL GLYCEROL TEICHOIC ACID**

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Antisera prepared by immunization of rabbits with heat-killed group A streptococcal vaccines often contain precipitating antibodies to a soluble streptococcal antigen which has been identified as polyglycerophosphate.[1] Similar substances reacting with the same antisera are found not only in many different serological groups of streptococci but also in a variety of other Gram-positive species. Purified

preparations of polyglycerophosphate (PGP) from group A streptococci were nitrogen-free and appeared to be simple polymers of glycerol phosphate.[1] However, it is evident that this antigen belongs to the class of substances, isolated by Baddiley and his co-workers from various Gram-positive bacterial species, which are designated as glycerol teichoic acids and which commonly possess ester-linked D-alanine and sugars as part of the molecule.[2] The absence of D-alanine in the streptococcal PGP has been ascribed to the extreme lability of the ester linkage with resultant loss of this component during purification procedures.

In a recent study of cellular antigens of group A streptococci, Wilson and Wiley have described the occurrence of an antigen, designated E_4, that is clearly related to but distinguishable from PGP.[3] E_4 migrates more slowly than PGP on immunoelectrophoresis and gives precipitin reactions with certain streptococcal antisera that fail to react with PGP. The relatedness of the two antigens is demonstrated by certain other antisera that react with both antigens and give lines of identity on immunodiffusion. The present paper shows that the difference between E_4 and PGP depends upon the presence of a heavy complement of ester-linked D-alanine in the former antigen, which is accordingly abbreviated as Ala-PGP. Furthermore, it is established that D-alanine is the major determinant of serological specificity of this antigen.

Materials and Methods.—Streptococcal strains: Although a variety of strains of group A streptococci were used in the course of these studies, most of the preparative work was carried out with either T4/95 (type 4) or T27A/32 (group A-variant, type 27).

Antisera: The antisera were obtained in the course of the routine production of grouping and typing sera by the intravenous injection of heat-killed streptococcal vaccines into rabbits. These sera were prepared chiefly with group A strains, although a group G antiserum contained the highest concentration of antibodies to E_4. Additional E_4 antisera were generously provided by Dr. Armine T. Wilson.

Polyglycerophosphate: This antigen was isolated from extracts of streptococci obtained by boiling at pH 2 as previously described.[1]

Preparation of E_4 antigen (Ala-PGP): As pointed out by Wilson and Wiley,[3] E_4 is found in extracts of streptococci obtained by a wide variety of different procedures. The antigen is labile, however, and extracts lose activity with time. Because of this lability and the difficulty of separating the antigen from ribonucleic acid, a method of extraction was selected which could be carried out rapidly with a minimum of contamination with other cellular constituents. The procedure described depends on the fact that approximately one half of the total antigen in the cells is obtained on simple extraction with buffer solutions.

In a typical preparation, 30 liters of Todd-Hewitt broth, previously warmed to 37°C, were inoculated with 2 liters of a culture of strain T27A grown 16 hr at room temperature. After 4.5 hr incubation at 37°C, the young bacterial cells were collected in a Sharples centrifuge. The cells were washed by resuspension in 200 ml 0.2 *M* acetate buffer pH 6.0 and immediately recentrifuged. They were then extracted repeatedly with 200-ml portions of the same buffer by stirring at 37°C for 30 min followed by centrifugation. The second and third extracts, which contained the bulk of the antigen as indicated by serological test, were pooled, chilled to 4°C, and mixed with 2 volumes of cold absolute ethanol. After refrigeration overnight, the precipitated antigen was recovered by centrifugation, redissolved in 20 ml acetate buffer, and the insoluble residue discarded. The ethanol precipitation was repeated, the precipitate redissolved in 10 ml acetate buffer, and the small amount of insoluble material removed by centrifugation. The solution was dialyzed overnight in the cold against acetate buffer, reprecipitated with ethanol, and finally dried from absolute ethanol and ether. The yield was 41 mg.

Ala-PGP obtained by this simple procedure contained no other recognizable streptococcal antigens and, on the basis of UV absorption at 260 mμ, less than 0.5% nucleic acid.

Quantitative precipitin tests: These tests were carried out with 0.1- or 0.2-ml amounts of anti-

serum in a final volume of the reaction system of 1.0 ml. The diluent employed was 0.2 *M* acetate buffer, pH 6.0. After refrigeration overnight, the precipitates were washed three times in 3.0-ml portions of the same buffer, redissolved in 2.0 ml 0.1 *N* NaOH, and optical density determined at 287 mμ in the Beckman spectrophotometer.

Chemical determinations: Total phosphorus was determined by the micro method of Chen *et al.*[4] Alanine content was estimated by quantitative paper chromatography; the results were confirmed for one sample by ion exchange chromatography carried out by Dr. T.-Y. Liu with the automatic recording equipment described by Spackman, Stein, and Moore.[5] The configuration of the alanine was determined by the D-amino acid oxidase method using enzyme from hog kidney, prepared by the method of Massey, Palmer, and Vennett.[6]

Results.—Chemical properties of Ala-PGP: The sodium salts of the Ala-PGP, prepared as described in *Materials and Methods*, contained 12.5–13.5 per cent phosphorus. All of the N was accounted for as alanine, with a molar ratio P:N ranging from 1.75 to 2. Analytical data on three different purified lots obtained from two strains of group A streptococci are given in Table 1.

It is probable that some of the labile ester-linked alanine was lost even in the relatively rapid purification described. For example, pilot lots prepared from smaller batches of organisms so that more rapid handling was possible were found to have P:N ratios below 1.5. Thus, the alanine complement of the intact antigen as it exists in the cell may more nearly approach one amino acid residue for each glycerophosphate unit. The alanine released by acid hydrolysis was totally digested by D-amino acid oxidase indicating that it is all present in the D-configuration.

Serological reactivity of Ala-PGP: Acetate buffer extracts of young streptococcal cells react promptly in precipitin tests with antisera of the E_4 type (Wilson and Wiley[3]), but show little or no reaction with PGP antisera. However, the cross-reactivity of material obtained during purification usually increased slightly at each step, suggesting that the loss of labile D-alanine might be responsible. The precipitates formed in the cross-reaction with PGP antisera tended to develop slowly and to increase with time even at refrigerator temperatures. Since most of the rabbit antisera employed have a pH of 8.3 or above, the possibility arose that the alkaline conditions of the precipitin test were promoting cross-reactivity by enhancing the hydrolysis of alanine esters. This possibility was substantiated by demonstrating that the use of sera which had been dialyzed against pH 6 acetate buffer greatly reduced the cross-reaction of Ala-PGP preparations with PGP antisera without affecting the reactivity with homologous antisera.

The serological interrelationships between Ala-PGP and PGP are best illustrated by intentional removal of the ester-linked D-alanine. If solutions of Ala-PGP are adjusted to pH 9 at room temperature or are heated at 100°C even at pH 6, there is a progressive loss of reactivity with Ala-PGP sera and a concomitant increase in reactivity with PGP antisera. The only chemical change that can be detected during this treatment is the release of free alanine. These relationships are illustrated in the experiment summarized in Figure 1.

TABLE 1

ANALYSES OF ALA-PGP FROM GROUP A STREPTOCOCCI

Preparation	P, μmole/mg	D-alanine, μmole/mg
T4 lot 1	4.3	2.2
T27A lot 11–63	4.32	2.03
T27A lot (2 + 3)	4.03	2.28

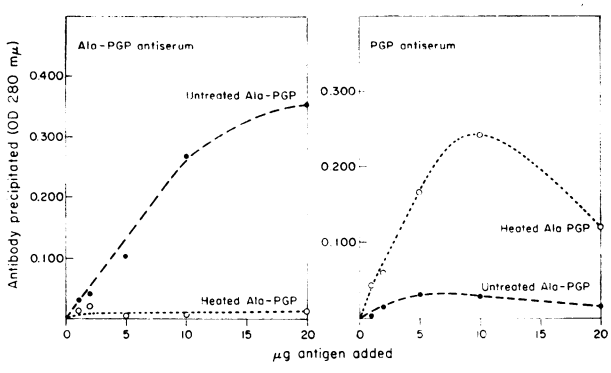

FIG. 1.

A solution containing 1 mg/ml of Ala-PGP in water was mixed with an equal volume of 0.02 *N* NaOH, heated in a boiling waterbath for 5 min, neutralized, and diluted to a final concentration of 0.1 mg/ml in 0.2 *M* acetate buffer, pH 6. Quantitative precipitin curves comparing the treated and untreated material were carried out in both Ala-PGP and PGP antisera. Figure 1 shows that the reactivity of the antigen with the Ala-PGP antiserum was completely destroyed by the brief period of heating in dilute alkali. On the contrary, the weak cross-reactivity of the antigen with PGP antiserum was markedly enhanced by this treatment, so that the material developed reactivity comparable to stock preparations of PGP.

Role of D-*alanine in serological specificity of Ala-PGP:* The foregoing data indicate that the ester-linked D-alanine must contribute an important element of the serological specificity of Ala-PGP and at the same time must suppress the expression of glycerophosphate specificity. Synthetic polyglycerophosphate with an average chain length of 6 units,[7] which is a highly potent inhibitor of the reaction of PGP with its homologous antisera,[1] is without significant effect on the precipitation of Ala-PGP with its antisera. This confirms the limited role that the basic glycerophosphate structure plays in the latter reaction.

D- and L-Alanine and D- and L-*N*-acetylalanine show no inhibitory effect on the Ala-PGP precipitin reaction at concentrations of 0.01–0.05 *M*. However, the methyl esters, with configurations presumably more comparable to that of the D-alanine present in the antigen, show specific inhibition with a variety of different Ala-PGP antisera. Table 2 records the per cent inhibition observed with four different antisera in quantitative precipitin tests carried out at the equivalence point in the presence of the methyl esters of alanine at a final concentration 0.05 *M*. The D-alanine methyl ester consistently caused greater inhibition than the L isomer.

The D-alanine methyl ester causes a retardation in the rate of precipitate forma-

TABLE 2

SPECIFIC INHIBITION OF ALA-PGP PRECIPITIN REACTION WITH ALANINE METHYL ESTERS

Inhibitor	Antisera			
	R594	R766	R1351	R1642
	Per cent inhibition			
L-Alanine methyl ester	31	28	23	20
D-Alanine methyl ester	48	85	40	35

Reaction mixture at equivalence point in final volume at 1.0 ml. Inhibitors at final concentration 0.05 *M*.

tion that is even more striking than the decrease in the amount of final precipitate formed. The experimental tubes containing this inhibitor frequently remained almost clear at a time when maximal flocculation had occurred in control tubes. An attempt to illustrate this behavior by carrying out the precipitin reactions in Beckman cuvettes and reading turbidity at 450 mμ is summarized in Figure 2. The proportions of antigen, antibody, and inhibitor are the same as in the experiments shown in Table 2. The rapidity of the precipitate formation is evident, as is the superiority of D-alanine methyl ester over the L-derivative as an inhibitor. The subsequent decrease in turbidity with time after the maximum is reached reflects the tendency of the precipitates to aggregate promptly into coarse floccules which were not altogether dispersed by stirring before each reading.

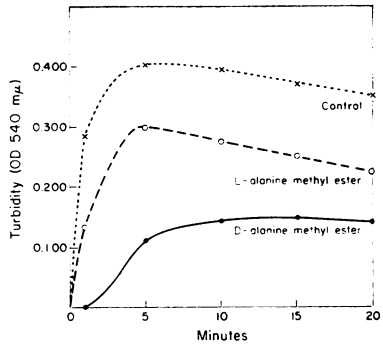

FIG. 2.

Interrelationships between PGP and Ala-PGP: Although many antisera distinguish sharply between nitrogen-free PGP and the alanine-containing preparations, certain rabbits immunized with streptococci produce antisera that react about equally well with either antigen. In the case of many antisera of this latter type, it was found by Wilson (personal communication) and confirmed in this laboratory that absorption with PGP removes all antibodies reactive with either antigen. This can only be explained by assuming that the antibodies reactive with Ala-PGP in these sera are directed against glycerophosphate determinants which are not effectively masked by the D-alanine residues. The fact that small molecular synthetic polyglycerophosphate strongly inhibits (90%) the reaction of Ala-PGP with these antisera substantiates this point of view. Furthermore, the alanine methyl esters are without inhibitory effect in this situation. It would appear, therefore, that the different types of antibody formed in rabbits in response to glycerophosphate determinants vary in the degree to which alanine esters interfere with combination with the antigen. The antigen preparations used here have about one half of the hydroxyls on carbon 2 of the glycerol units esterified with alanine, and stable preparations with heavier alanine complements have not been available to determine whether masking will be more complete under these conditions.

Discussion.—The distribution in the bacterial world of glycerol teichoic acids composed solely of glycerophosphate and D-alanine is not thoroughly established. As noted previously, extracts of most streptococci as well as of a number of other Gram-positive organisms contain antigens with glycerophosphate specificity. It became evident in the course of this study that these same organisms for the most part produce antigens which react with antisera possessing specificity for the D-alanine esters. However, the possibility exists that these may represent cross-reactions with certain of the more complex glycerol teichoic acids possessing substituent saccharide residues as well as ester-linked D-alanine. The group-specific antigen of group D streptococci, which is a glycerol teichoic acid with di- and trisaccharides of glucose attached to the glycerol,[8] has been shown by Elliott to cross-

react weakly with PGP antisera at high antigen concentrations.[9] On the other hand, PGP does not react with group D antisera.

In the case of group N streptococci the group-specific antigen is a glycerol teichoic acid containing galactosyl residues which also reacts with PGP antisera.[10] However, some strains of group N also appear to produce a polyglycerophosphate polymer without galactosyl substituents.

Certain other purified preparations, such as the intracellular teichoic acids from lactobacilli,[11, 12] have not been tested for reactivity with the streptococcal antisera.

The major contribution of ester-linked D-alanine to the serological specificity explains the serologic and immunoelectrophoretic behavior of the E_4 antigens as initially described by Wilson and Wiley.[3] Since the amino group of the alanine residues is free, the net negative change of Ala-PGP (or E_4) is considerably less than that of PGP and thus the electrophoretic mobility toward the anode is decreased. Ala-PGP is able to react with both types of antisera, and its cross-reactivity with PGP antisera tends to increase as D-alanine is lost by hydrolysis of the labile ester. On the other hand, PGP lacking D-alanine, can only react with antisera of glycerol phosphate specificity. When antisera are used which react with both Ala-PGP and PGP, the two antigens would be expected to give joining arcs on immunoelectrophoresis or immunodiffusion since the same determinant is involved in each case.

The fact that the D-alanine esters can act as antigenic determinants is of interest, because it had been concluded in the case of one of the ribitol teichoic acids of staphylococci that alanine residues play no part in the serological reactions.[13] It seems possible that under certain conditions one might obtain antisera to staphylococcal teichoic acid with a portion of the antibodies possessing D-alanine specificity.

Among the earliest studies on antigens now known to represent glycerol teichoic acids were those of Rantz *et al.*[14] who showed by passive hemagglutination techniques that a common heterophile antigen occurred in a variety of bacterial species. It has now been shown by inhibition studies that PGP is responsible in a large degree for the reactions observed.[15, 16] Thus, purified preparations of streptococcal PGP strongly inhibited the hemagglutin of erythrocytes coated with Rantz-type antigens, but PGP itself seems unable to coat erythrocytes. The work of Jackson and Moskowitz[17] suggests that the presence of D-alanine esters is required for attachment of the antigen to the red cell surface. Thus, the antigen involved in the hemagglutination reaction is Ala-PGP, probably with something less than a full complement of D-alanine residues, although the serological specificity of the reaction appears to be largely directed to the PGP moiety.

Summary.—Ester-linked D-alanine is an important determinant of serological specificity of the glycerol teichoic acid present in group A streptococci. The presence of D-alanine suppresses to some degree the reactivity of the underlying glycerophosphate determinants.

The author wishes to acknowledge the assistance and cooperation of Dr. Armine T. Wilson, who first brought the E_4 antigen to his attention and supplied materials and information for the initiation of this study. The author is indebted to Dr. T.-Y. Liu for the chromatographic analysis and the D-amino acid oxidase assay.

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THE CAPSULAR POLYSACCHARIDE OF A MUCOID VARIANT OF E. COLI K12

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The isolation and properties of a polysaccharide elaborated by a mucoid variant of the colicine K-producing bacillus *E. coli* K235 were described in a previous communication.[1] This substance, named colanic acid, encapsulates the bacillus and is antigenic in rabbits. There are many accounts in the literature of mucoid forms of *E. coli* and of other Enterobacteriaceae as well. In certain instances these variants have a special encapsulating antigen which has been termed the M antigen.[2] It has been suggested by Kauffmann that this substance, regardless of the microorganism which elaborates it, is in all instances the same or very nearly so. Many investigators have reiterated this concept, yet their evidence in support of it has for the most part been fragmentary.

During the course of an investigation on bacteriophage T6, it was observed that when cultures of *E. coli* K12W were plated with an excess of virus, resistant mucoid forms of the host cell developed. We became curious as to the nature of the substance which endowed these cells with their remarkable mucoid characteristics. From that which follows, it will be seen that this material is to all intents identical with the colanic acid elaborated by the colicinogenic bacillus *E. coli* K235(m).

Materials and Methods.—Bacterial strains: Two strains of *E. coli* were used in this study. One was the mucoid variant of the colicine K-producing bacillus *E. coli* K235 L+O,[1, 3] the other a mucoid variant of *E. coli* K12W 1485 picked from a plate which had been seeded with 5×10^7 bacilli and 2×10^8 T6 phage particles, then incubated for 24 hr.

Identification of sugar components of K12 polysaccharide: A sample of the K12 polysaccharide (25 mg) was hydrolyzed in 1 *N* H_2SO_4 at 100°C for 4 hr. The hydrolysate was freed of sulfate ions, and the solution concentrated *in vacuo* (40 mg/ml); 0.005 ml containing 200 γ of hydrolysate was then chromatographed on Whatman no. 1 paper. For purposes of comparison, a similar hydrolysate of colanic acid was prepared and spotted on the same paper, together with samples of glucose,