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Nitrogen-Containing and Carbohydrate-Containing Antigen from *Actinomyces bovis*

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ABSTRACT

PIRTLE, E. C. (National Animal Disease Laboratory, Ames, Iowa), P. A. REBERS, AND W. W. WEIGEL. Nitrogen-containing and carbohydrate-containing antigen from *Actinomyces bovis*. J. Bacteriol. **89**:880–888. 1965.—Water-soluble, heat-stable antigens have been isolated from the supernatant fluids of broth cultures of *Actinomyces bovis* ATCC 10048 after 8 days of growth in a broth medium composed of Casamino Acids, yeast extract, Tween 80 (polyoxyethylene sorbitan monooleate), sodium thioglycolate, dextrose, and sodium chloride. The antigens were precipitated from the culture supernatant fluid with alcohol and purified by fractional precipitation with alcohol in the presence of calcium or zinc and by chromatography on diethylaminoethyl Sephadex. Two serologically active substances which differed in chemical composition and size were characterized. The larger one contained 71% hexose and 2.8% nitrogen, and the smaller one contained 45% hexose and 5.4% nitrogen. Heterogeneity of these fractions could not be demonstrated by electrophoresis in free solution at *p*H 7.8 or 2, by ultracentrifugal analysis, by double diffusion in agar, or by immunoelectrophoresis. Despite their differences in chemical composition and size, they appeared identical in activity as antigens in complement fixation and in double-diffusion tests in agar. Each was found to contain mannose as its chief component after hydrolysis and paper chromatography with three solvent systems. A small percentage of the total nitrogen may be attributed to the presence of amino sugars but the remainder is as yet unidentified.

Since Wolff and Israel (1891) first isolated *Actinomyces bovis* in pure culture, only a few reports have appeared concerning the antigenic composition of this organism. Most of the past research has been directed toward the separation of different strains or serological groups of *A. bovis* on the basis of agglutination reactions or fluorescent-antibody studies (Colebrook, 1921; Magnusson, 1928; Aoki, 1936; Erickson, 1940; Slack et al., 1955; Slack, Winger, and Moore, 1961), rather than toward characterization of antigenic components of single strains. Goyal (1938) prepared complement-fixing antigens by methanol extraction of unemulsifiable cells from strains of *Actinomyces*, but the organisms were not identified as to species or growth requirements. King and Meyer (1963) recently described precipitating antigens of *Actinomyces* species prepared from supernatant infusion broth culture fluids by acetone precipitation in the cold. Multiple precipitin bands were produced in agar-gel diffusion plates when antigens of *Actinomyces* species were tested with homologous rabbit antisera. *A. israelii* was found to be serologically distinct from *A. bovis*, but *A. naeslundii* shared a common antigen with both of these species.

Preliminary studies (Weigel, 1960) relative to the present report indicated that supernatant fluids from sonically treated cells and broth cultures of *A. bovis* contained complement-fixing and precipitating antigens which reacted with homologous immune rabbit serum. Although cell debris obtained from sonically treated cells produced precipitin lines in agar with homologous rabbit antiserum and elicited delayed skin reactions in sensitized rabbits, it proved to be grossly anticomplementary.

Since the preliminary research was carried out, purification of the reactive complement-fixing and precipitating antigen from cell-free supernatant fluids from *A. bovis* cultures has been achieved. The present report describes the preparation of the complement-fixing and precipitating antigen of *A. bovis* and presents evidence for its chemical nature and properties.

MATERIALS AND METHODS

Inoculum. A. bovis ATCC 10048 (*A. israelii*) was used throughout these studies.

Medium. The broth medium used for propagation of *A. bovis* had the following composition: 1.0% Casamino Acids (Difco), 0.2% dextrose, 0.3% yeast extract, 0.2% NaCl, 0.1% sodium thioglycolate, and 0.02% Tween 80 (polyoxyethylene sorbitan monooleate). The medium was sterilized by autoclaving at 121 C for 30 min.

Preparation of antiserum. Organisms from 8-day broth cultures were washed three times with sterile buffer containing 0.137 M NaCl, 0.01 M Na_2HPO_4, and 0.003 M KH_2PO_4 (*p*H 7.2). The vaccine contained 5×10^6 viable organisms per milliliter as determined by plate counts. Albino rabbits were given an initial series of five intravenous injections at 4- or 5-day intervals of 0.1, 0.2, 0.4, 0.6, and 1.0 ml. Booster injections of 1.0 ml and 0.5 ml were given 42 and 183 days, respectively, after the initial series was completed. Vaccine for booster injections was prepared as above. Final bleedings were carried out 7 months after injections were begun. Two of the rabbit antisera with equivalent complement-fixation titers were pooled as reference antiserum RA I-4. One other rabbit antiserum, S-V, was used in some of the agar double-diffusion tests.

In addition to the sera listed above, John M. Slack kindly provided us with two *A. bovis* antisera from his serological groups C and D. *A. bovis* ATCC 10048, employed in the present study, has been designated as belonging to group D (Slack et al., 1961).

Preparation of antigen. A 14-liter amount of the Casamino Acids-thioglycolate medium was inoculated with 20 ml of an actively growing *A. bovis* culture and incubated for 8 days at 37 C. Cultures were agitated daily during the period of incubation to resuspend the organisms and to minimize clumping. The organisms were removed by centrifugation at 18,000 rev/min in a Servall centrifuge (SS-34 head) with a continuous-flow attachment. The supernatant fluid was treated with 10 g of C_6H_5OH and several drops each of $CHCl_3$ and $C_6H_5CH_3$; then 1,300 g of $NaC_2H_3O_2$ and 400 ml of 25% CH_3COOH were added; final *p*H was 5.0. The mixture was cooled to 4 C, and 10 volumes of 95% C_2H_5OH were added. A light-tan precipitate settled out after 1 day. After 2 weeks, the supernatant fluid was removed by centrifugation and discarded. The precipitate was dissolved in 130 ml of distilled water and mixed with 400 ml of 0.7 M acetate buffer (*p*H 5.2); 2 liters of cold 95% C_2H_5OH were added with stirring, and a flocculent precipitate was formed. The serologically active crude material precipitated completely in 3 days at 4 C. The crude antigen was fractionated by precipitation with C_2H_5OH, CH_3OH, and by column chromatography on diethylaminoethyl (DEAE) Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden). All alcohol fractionations were carried out by the dropwise addition of either C_2H_5OH or CH_3OH until a faint turbidity was first noted. At this point, precipitation was allowed to proceed for at least 24 hr before removal of each precipitate by centrifugation.

Nonspecific control antigen for serological tests was prepared by dialyzing and concentrating uninoculated broth medium of the same type used for propagation of *A. bovis* cultures; the medium was concentrated 10 times by pervaporation.

Paper chromatography. Descending paper chromatography was carried out at 23 C with Whatman no. 1 paper with the following solvent systems: butanol-pyridine-water, 6:4:3 (Chargaff, Levine, and Green, 1948); phenol saturated with water (Partridge, 1948); butanol-ethyl alcohol-acetic acid-water, 100:50:10:20 (Kwapinski, 1960).

Sugars were located on chromatograms with aniline-trichloroacetic acid (McCready and McComb, 1954), and with the alkaline $AgNO_3$ procedure of Trevelyan, Procter, and Harrison (1950) modified so that a dipping reagent of 0.5 N KOH in equal parts of 95% C_2H_5OH and $CH_3CHOHCH_3$ replaced a spray of 0.5 N NaOH in 95% C_2H_5OH.

Column chromatography. Column chromatography of the antigen was performed with DEAE Sephadex A-50. It was chosen rather than DEAE cellulose because it has less tendency to contaminate the eluates with soluble carbohydrate impurities arising from partial breakdown of the exchange medium (Huffman et al., 1955). Before use, the DEAE Sephadex was suspended in distilled water and the fines were poured off. The material was washed with 1 N HCl, 1 N NaOH, and distilled water; CO_2 was bubbled through the suspension to a *p*H of 7.2 to 7.6.

Analytical methods. Nitrogen was determined by a micro-Kjeldahl method (Kabat and Mayer, 1961).

Carbohydrates were determined by the phenol-sulfuric acid method (Dubois et al., 1956), and by a modification of this method in which 5 ml of concentrated H_2SO_4 were added to 2 ml of aqueous solution of the sample, and absorbancy was measured at 290 mμ (Ikawa and Niemann, 1949). The primary cysteine-sulfuric acid reaction of hexoses was also employed (Dische, 1955). All absorbancies were measured with a Zeiss PMQ II spectrophotometer.

Total phosphate was determined after digestion with H_2SO_4 and H_2O_2 with a procedure using *p*-semidine as the reducing agent (Dryer, Tammes, and Routh, 1957).

Assays for sialic acid were made by the thiobarbituric acid method of Warren (1959).

Tests for esters and lactones were carried out by use of the hydroxamic acid reaction (Abdel-Akher and Smith, 1951).

The carbazole reaction was used to detect the presence of uronic acids in unhydrolyzed polysaccharide fractions (Dische, 1955).

Assays for amino sugars were carried out after 2-hr hydrolysis with 5 N HCl by a modified Elson-Morgan method (Kabat and Mayer, 1961), and with a method involving deamination followed by reaction with indole (Dische, 1955).

Microscale electrophoresis in free solution was performed with the apparatus described by Antweiler (1949).

The Spinco model E analytical centrifuge was used to test purified antigens for homogeneity and to determine sedimentation coefficients both with the schlieren and ultraviolet-absorption optical systems (Schachman, 1957).

Immunoelectrophoresis. Immunoelectrophoresis was done on microscope slides in an Agafor apparatus (National Instrument Laboratories, Inc., Washington, D.C.). A 0.9% solution of agarose (Marine Colloids, Springfield, N.J.) in tris(hydroxymethyl)aminomethane (Tris)-citrate buffer, *p*H 8.6 (Matson, 1962), was employed as the diffusion medium, 4 ml per slide. Electrophoresis of antigen was carried out for 40 min at 75-ma constant current prior to the addition of antiserum.

Antigen titration and complement-fixation tests. Titrations were carried out by the block method, reacting increasing dilutions of immune serum against increasing dilutions of the antigen. The complement-fixation test described by Schmidt and Lennette (1955) was employed to determine complement-fixing titers of the rabbit antisera, except that Veronal buffer (Kabat and Mayer, 1961) was used. Appropriate controls of all reactants were included with each series of tests.

Double-diffusion tests in agar. The agar formula contained 0.6% Ionagar No. 2 (Consolidated Laboratories, Inc., Chicago Heights, Ill.) dissolved in 0.1 M NaCl in 0.05 M Tris buffer of *p*H 7.3. Merthiolate (Eli Lilly & Co., Indianapolis, Ind.) was added as a preservative in a final concentration of 1:10,000. Ouchterlony plates were prepared by cutting wells in solidified agar with Feinberg cutter 1802, or cutter 1815 modified to hold a no. 1 cork borer in its center position. Plates were incubated at 22 C for 3 to 7 days before being photographed.

TABLE 1. *Properties and serological activities of ethyl alcohol fractions of crude antigenic material obtained from cell-free supernatant fluids of Actinomyces bovis cultures*

Fraction	Vol of C_2H_5OH added	Yield of precipitate[a]	Specific rotation $[\alpha]_D^{22}$	Total nitrogen	Hexose[b] (phenol method)	Serological activity	
						Complement-fixation titer	Ouchterlony[c]
	ml	*g*		%	%		
P-1	100	1.1				8[d]	50
P-2	200	2.3					20
P-3	300	0.31	+35°	3.2	85	128	1
P-4	400	0.28	+20°	4.8	50	128	1

[a] Total weight of nondialyzable solids after dialysis and lyophilization.

[b] Calculated by comparison with a standard of mannose.

[c] Activity of the fraction expressed as the minimal concentration in milligrams that yielded a precipitin line with rabbit antiserum RA I-4.

[d] Reciprocal of antigen dilution containing one unit of antigen, determined by block titration. Activity based on 1 mg/ml of undiluted antigen.

TABLE 2. *Properties and serological activities of subfractions of fractions P-3 and P-4 obtained by precipitation with methanol in the presence of calcium acetate*

Fraction	Vol of CH_3OH added	Yield of precipitate	Specific rotation $[\alpha]_D^{22}$	Total nitrogen	Hexose[a] (phenol method)	Serological activity[b]	
						Complement fixation titer	Ouchterlony[c]
	ml	*mg*		%	%		
P-3-1	9	35	+4°				Inactive
P-3-2	17	27					Active
P-3-3	23	159	+97°	3.1	69	128[d]	Active
P-3-4	43	47					Inactive
P-4-1	4	66	0°				Inactive
P-4-2	12	38	0°				Active
P-4-3	28	61	+26°	4.2	58	128	Active
P-4-4	48	83					Inactive

[a] Calculated by comparison with a standard of mannose.

[b] All samples prepared in a concentration of 1 mg/ml.

[c] Precipitin activity with rabbit antiserum RA I-4.

[d] Reciprocal of antigen dilution containing one unit of antigen, determined by block titration.

RESULTS AND DISCUSSION

Ethyl alcohol, sodium acetate fractions. The relatively crude antigenic material obtained by C_2H_5OH precipitation as described in Materials and Methods was removed by centrifugation, dissolved in 250 ml of a solution of a 0.67 M acetate buffer (*p*H 5.0), and fractionated by the stepwise addition of C_2H_5OH as shown in Table 1. Fractions P-3 and P-4, which amounted to only one-sixth of the total solids of the starting material, contained most of the serological activity. Although the composition of these two fractions was significantly different, no differences in their complement-fixing and precipitating activities could be detected.

Subfractionation with methanol and calcium acetate. Fractions P-3 and P-4 (Table 1) were each dissolved in 12 ml of 0.5 M calcium acetate buffer (*p*H 4.9). Further fractionation of each solution was carried out by the stepwise addition of CH_3OH (Rebers and Heidelberger, 1959) as shown in Table 2. The resulting fractions were screened for serological activity by testing them in Ouchterlony plates. Only fractions P-3-2, P-3-3, P-4-2, and P-4-3 formed a precipitin line with rabbit antiserum RA I-4. Fractions P-3-3 and

P-4-3 also had complement-fixing activities equivalent to the C_2H_5OH fractions from which they were obtained. Since fractions P-3-2 and P-4-2 were not completely soluble in water, they were not included in subsequent experiments. The fractions which were inactive with antiserum RA I-4 did show slight precipitin activity with Slack's group D antiserum.

Subfractionation of P-3-3 with methanol and zinc acetate. A 36-ml amount of a 0.5 M solution of $Zn(C_2H_3O_2)_2$, *p*H 5.9 (Rebers and Heidelberger, 1959), was added to 154 mg of subfraction P-3-3. On the addition of 59 ml of CH_3OH, 13 mg of precipitate, P-3-3-1, were obtained; an additional 40 ml of CH_3OH precipitated 68 mg, P-3-3-2. The latter formed a precipitin line and reactions of identity at a concentration of 1 mg/ml with antisera RA I-4, S-V, and Slack's group D, but not with Slack's group C (Fig. 1). Precipitate P-3-3-1 was serologically inactive under the same conditions.

Column chromatography of partially purified and crude fractions. A 56-mg amount of subfraction P-4-3 was dissolved in distilled water and added to a column (8 by 24 mm) of DEAE Sephadex A-50. Successive elutions with 20-ml volumes of distilled water, and with 0.0075, 0.03, 0.15, and 2.9 M NaCl yielded fractions containing 18.4, 3.3, 0.3, 25.4, and 4.6 mg, respectively, of nondialyzable solids. The 0.15 M NaCl eluate, P-4-3-DEAE, was the only one that formed a precipitin line in a concentration of 1 mg/ml with antiserum RA I-4 (Fig. 2).

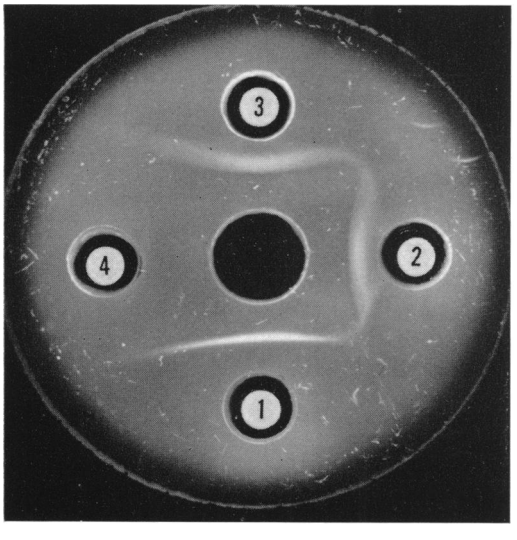

FIG. 1. *Precipitin reactions between Actinomyces bovis subfraction P-3-3-2 and A. bovis rabbit antisera. Central well, subfraction P-3-3-2. Outer wells: (1) Slack's group D antiserum; (2) RA I-4 antiserum; (3) S-V antiserum; (4) Slack's group C antiserum.*

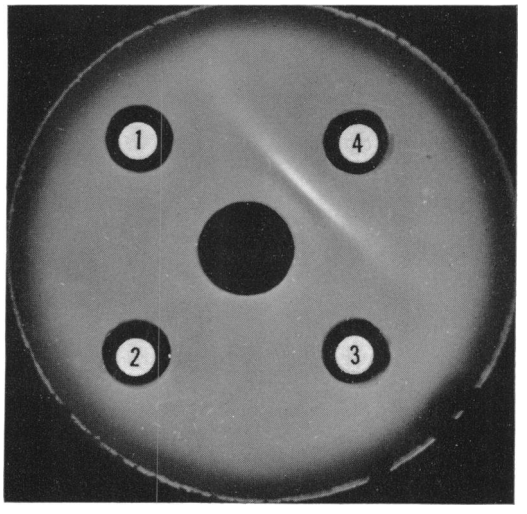

FIG. 2. *Precipitin reactions between the eluates from subfraction P-4-3 obtained by column chromatography on DEAE Sephadex A-50 and antiserum RA I-4. Central well, RA I-4 antiserum. Outer wells: (1) distilled water eluate; (2) 0.0075 M NaCl eluate; (3) 0.03 M NaCl eluate; (4) 0.15 M NaCl eluate (fraction P-4-3-DEAE).*

A 222-mg portion of crude fraction P-2 was fractionated on DEAE Sephadex A-50 in a similar manner, except that 1.5 M NaCl in 0.017 M CH_3COOH was used in addition to the other eluants. Inactive material, 178 mg, was obtained with all eluants up to 1.5 M NaCl; 11 mg of active material (fraction P-2-DEAE) were eluted by the latter eluant. The active material at a concentration of 6 mg/ml formed four precipitin lines with Slack's group D antiserum but only one with antiserum S-V (Fig. 3 and 4). It is obvious that these two antisera differed in potency and perhaps in specificity. However, when Slack's antiserum was diluted 1:3 it also yielded only one precipitin line. Results obtained from column chromatography of a second 200-mg sample of P-2 corroborated the initial findings.

About 10 mg of subfraction P-3-3-2 had the same elution characteristics from DEAE Sephadex A-50 as crude fraction P-2.

The elution pattern of fraction P-4-3 which required 0.15 M NaCl and the two other fractions which required 1.5 M NaCl for elution is comparable to the gradient-elution chromatogram of bovine vitreous humor on DEAE Sephadex A-50 (Berman, 1962). Three hyaluronic acid peaks were obtained, two of which differed

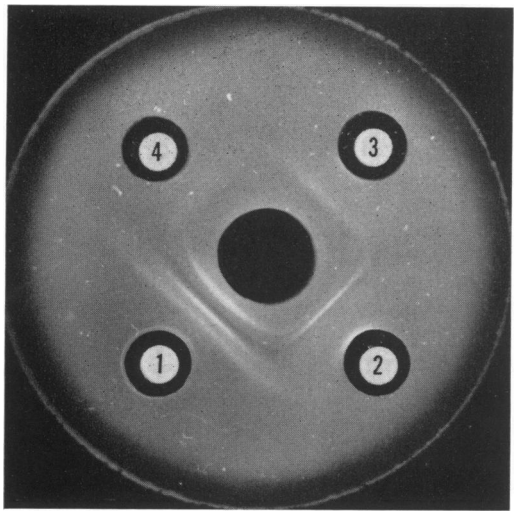

FIG. 3. *Precipitin reactions between Slack's group D antiserum and four different concentrations of the 1.5 M NaCl eluate from column chromatography of fraction P-2 on DEAE Sephadex A-50. Central well, Slack's group D antiserum. Outer wells: (1) 6 mg/ml; (2) 1 mg/ml; (3) 0.5 mg/ml; (4) 0.25 mg/ml.*

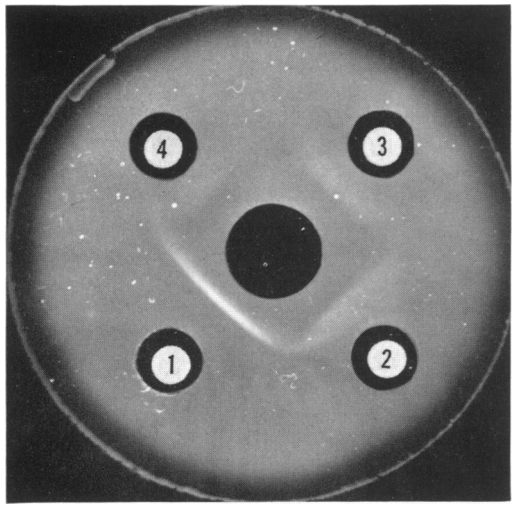

FIG. 4. *Precipitin reactions between S-V antiserum and four different concentrations of the 1.5 M NaCl eluate from column chromatography of fraction P-2 on DEAE Sephadex A-50. Central well, S-V antiserum. Outer wells: (1) 6 mg/ml; (2) 1 mg/ml; (3) 0.5 mg/ml; (4) 0.25 mg/ml.*

markedly in their limiting viscosity number. Fraction P-4-3-DEAE which was eluted with the lower concentration of NaCl also had the lower sedimentation coefficient (Table 3).

Physical properties of purified antigen fractions. Fraction P-3-3-2 was subjected to electrophoresis in free solution at *p*H 7.8 in phosphate buffer with an ionic strength of 0.1, at 22 C. A single symmetrical peak was observed which moved toward the anode (Fig. 5a). This same fraction was also run at *p*H 2.1 in an HCl-KCl buffer of 0.1 ionic strength, but the peak failed to move with electrophoresis under these conditions (Fig. 5b). These findings suggest that the antigen assumed a negative charge at *p*H 7.8 and was neutral at *p*H 2.1. This behavior is typical of many polysaccharides containing carboxyl groups, and is in contrast to the usual amphoteric nature of proteins. Although nitrogen was present at relatively high concentrations in the antigen, it was apparently not free to assume a positive charge at *p*H 2.1.

Fractions P-3-3-2 and P-4-3-DEAE were subjected to ultracentrifugation to test for homogeneity and to determine their sedimentation coefficients with the schlieren optical system. No evidence for heterogeneity of P-3-3-2 could be detected by this technique (Fig. 6), because only a single symmetrical peak could be seen at any time during the run. Only a single symmetrical peak was likewise obtained with fraction P-4-3-DEAE. However, the sedimentation coefficients,

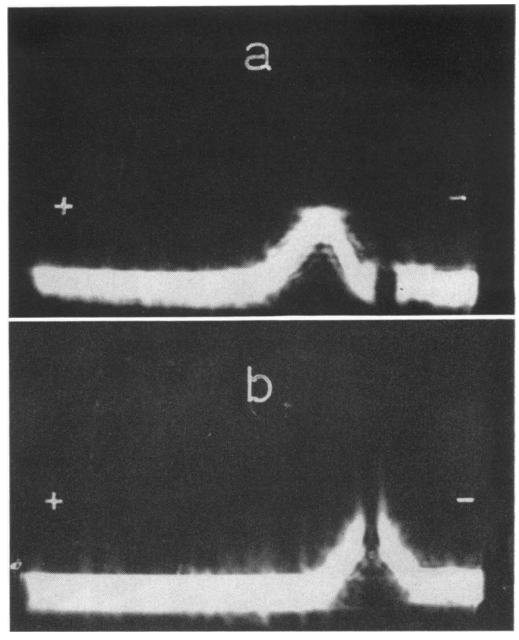

FIG. 5. *Electrophoretic patterns in free solution of Actinomyces bovis fraction P-3-3-2 at two pH levels: (a) migration at pH 7.8; (b) no migration at pH 2.1.*

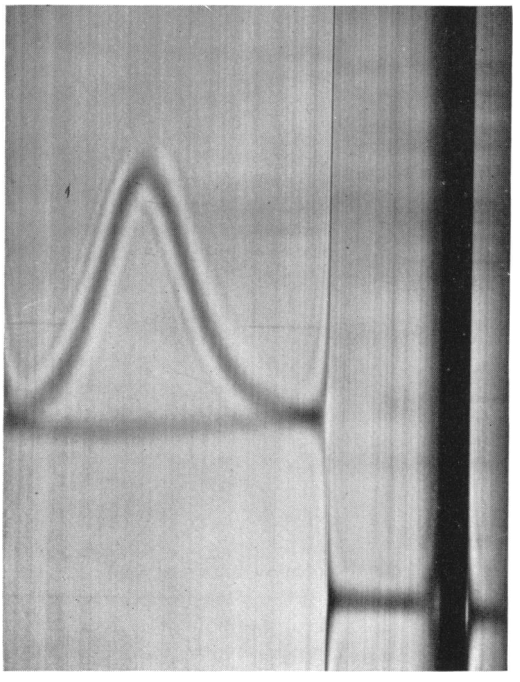

FIG. 6. *Schlieren sedimentation pattern taken at a bar angle of 40 with a double-sector cell of fraction P-3-3-2 at a concentration of 11.9 mg/ml in 0.2 M NaCl at 50,740 rev/min, 20 C, 201 min after start.*

as well as specific rotations, were significantly different (Table 3).

The absorbancy of both fractions at a concentration of 1 mg/ml in distilled water was measured between 230 and 300 mμ, in 10-mμ increments. A plateau was observed in both cases at 240 to 260 mμ, and a sharp increase below 230 mμ.

Since the antigen fractions absorbed in the ultraviolet region, a further attempt to detect heterogeneity in fraction P-3-3-2 was carried out in a model E Spinco centrifuge with an ultraviolet-absorption optical system. No evidence for heterogeneity was observed because the same sedimentation coefficient was obtained as with schlieren optics.

Stability of crude and purified antigens. No detectable change in the serological activity of crude antigen precipitated with C_2H_5OH occurred after autoclaving for 20 min at 121 C, or after storage for 2 years at 4 C and *p*H 7.2. Fraction P-3-3-2 retained its serological activity after 4 days at 22 C in 0.01 M phosphate buffer at *p*H 8.7. After heating at 80 C for 1 hr at *p*H 2.6 in 0.01 N H_2SO_4, most of the activity of P-4-3-DEAE was lost, and all activity was lost in 0.1 N H_2SO_4 at *p*H 1.6. Material balances before and after fractionation and dialysis of the purified antigens suggested that a portion of the product slowly dialyzed through Visking casing.

Chemical analysis of serologically active A. bovis fractions. Inasmuch as retention of the antigen fractions by DEAE Sephadex, an anion exchange polymer, as well as data on electrophoretic mobility, were indicative of the presence of carboxyl groups, tests were performed on fractions P-3-3 and P-4-3 for uronic acids with the carbazole reaction. Neither fraction gave the typical pink color characteristic of hexuronic acids. Examination of the wavelength absorbancy curve also failed to show any evidence of a peak at 540 mμ, which would indicate the presence of a uronic acid.

Assay of fractions P-2-DEAE and P-4-3-DEAE for sialic acid according to the thiobarbituric acid method gave no peak at 549 mμ, but a major peak was observed at 450 mμ and a minor peak at 524 mμ. Sialic acid (Warren, 1959) and ketodeoxyheptonic acid (Weissbach and Hurwitz, 1959) show absorption peaks at 550 mμ, the maximum for deoxyribose being at 530 mμ. A satisfactory interpretation of our results is lacking at this time, but the peak produced by P-4-3-DEAE, which had greater serological activity, was nine times higher than that produced by P-2-DEAE.

A test for esters or lactones by the hydroxamic acid test was negative on fraction P-2-DEAE.

Analysis of fraction P-3-3-2 for total phosphate gave a result of less than 0.01%.

On the basis of the previous experiments, it may be concluded that the acidic nature of the antigen is not due to the presence of uronic acids, sialic acids, or phosphate esters. However, Vi antigens, which contain 2-amino-2-deoxy-D-galacturonic acid, also fail to give a test for uronic acids (Heyns et al., 1959).

TABLE 3. *Comparison of the chemical and physical analyses of fractions P-3-3 and P-4-3 after further purification*

Analysis	Fraction	
	P-3-3-2	P-4-3-DEAE
Sedimentation coefficient, S_{20}	2.56	1.65
Specific rotation $[\alpha]_D^{22}$	+53°	+9°
Total nitrogen	2.8%	5.4%
Total phosphorus	None	
Hexose	71%	45%
Hydrolysis and paper chromatography	Mannose and trace of glucose	Mannose

To test for amino sugars, fractions P-3-3 and P-4-3 were hydrolyzed with 5 N HCl by heating at 100 C for 2 hr. The HCl was removed by evaporation in vacuo, and the residue was dissolved in distilled water. The hydrolysates were spotted on paper and sprayed with ninhydrin. Purple spots characteristic of amino derivatives were obtained in each case. Amino sugars were determined by the Elson-Morgan and Dische methods with glucosamine as a standard. Values of 3% were obtained with both procedures. Fractions P-3-3 and P-4-3 contained 3.1 and 4.2% nitrogen, respectively (Table 2). The nitrogen as "glucosamine" nitrogen in either case amounted to less than 10% of the total nitrogen. Therefore, unless the material is unusually resistant to, or is decomposed by, hydrolysis, most of the nitrogen is present in some form other than that of amino sugars reacting as glucosamine.

The primary cysteine reaction of hexoses is useful in determining the amount of pentose, hexose, heptose, methyl pentose, and deoxypentose in polysaccharides. Fraction P-4-3-DEAE had a typical mannose absorption curve 1 hr and 24 hr after the addition of cysteine. A typical mannose absorption curve also was obtained with the phenol-sulfuric method with fractions P-3-3-2 and P-4-3-DEAE (Fig. 7), except for the anomaly at the lower wavelengths with fraction P-4-3-DEAE. A modification of this reaction without the addition of phenol resulted in a wavelength absorption curve similar to mannose over the range of 220 to 350 mμ. No classes of sugars other than hexoses were found by any of these colorimetric reactions.

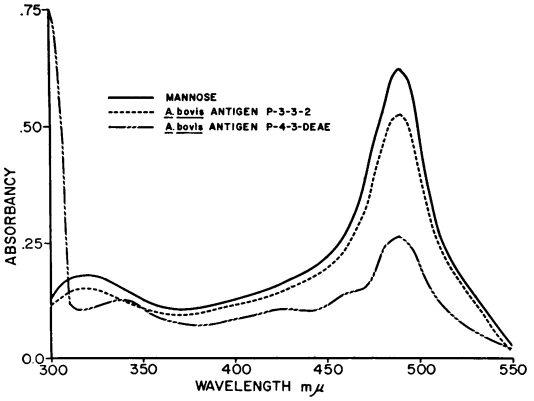

FIG. 7. *Spectrophotometric absorption curves of the chromogens obtained with the phenol-sulfuric acid reaction on the Actinomyces bovis antigens P-3-3-2 and P-4-3-DEAE compared with that of standard mannose.*

Hydrolysis and paper chromatography. Hydrolysis of fraction P-3-3-2 in 1.5 N H_2SO_4 at 100 C for 1.5 and 4.5 hr, followed by neutralization with $Ba(OH)_2$ and paper chromatography, showed no differences between the two hydrolysates. Fractions P-3-3-1 and P-3-3-2 gave spots corresponding to mannose and a trace of glucose, and the color of these with the aniline-trichloroacetic acid reagent corresponded to the brown color of the mannose standard rather than to the red of the arabinose standard. As mannose and arabinose yield the same R_F value in this solvent system, the run was repeated with the phenol-water system which readily separates the two, and the presence of mannose was again observed. Mannose was also observed in a third chromatogram with the butanol-ethyl alcohol-acetic acid-water solvent system. Fraction P-4-3-DEAE was found to contain only mannose. Extraction of the *A. bovis* cells with phenol in the cold (Westphal, Lüderitz, and Bister, 1952) yielded an aqueous layer which was also found to contain mannose, a trace of glucose, and a slow-moving unidentified component; this aqueous extract was serologically inactive with *A. bovis* antisera. Cummins and Harris (1958) identified galactose, glucosamine, and muramic acid as the carbohydrates in Mickle-treated, enzyme-digested cell-wall preparations of *A. bovis* 10048. In polysaccharide fractions of four strains of *A. israelii*, Kwapinski (1960) identified glucosamine and glucose (galactose) in all four and mannose in two of the strains; four other monosaccharides were also identified in some or all of these four strains. Georg, Robertstad, and Brinkman (1964) reported that monosaccharides were present in cell-wall preparations of four *Actinomyces* isolates, rhamnose in three, galactose and possibly glucose in two, and mannose in two; fucose was tentatively identified as another methyl pentose present in three of the isolates.

Possible linkages of mannose in the antigen. Inasmuch as the antigen was shown to contain mannose by paper chromatography, it seemed desirable to test its cross-reactivity with antisera known to react with mannans (Heidelberger and Cordoba, 1956). Michael Heidelberger tested fraction P-3-3-2 with antisera prepared against *Salmonella typhi*, *S. paratyphi* A, and *S. paratyphi* B. A definite cross-reaction was obtained with the anti-*S. typhi* and anti-*S. paratyphi* B sera, but not with anti-*S. paratyphi* A serum. To further investigate the possible linkages by which mannose is combined in the antigen, the same fraction was oxidized with periodate. In preliminary studies with $NaIO_4$ and fraction P-3-3-2, 1.5 moles of periodate and 0.3 mole of HCOOH

were produced per mole of mannose in the antigen after 3 weeks at 4 C; the oxidized product had no serological activity with RA I-4 antiserum. The serological and chemical data may be interpreted as indicating the presence of mannose joined in some combination of 1-2, 1-4, or 1-6 linkages, or possibly as end groups in the antigen.

Immunoelectrophoresis of fractions P-2-DEAE and P-4-3-DEAE. Single arcs of comparable mobility were obtained with the above two antigens and antiserum RA I-4; only the reaction of P-4-3-DEAE is shown (Fig. 8). Likewise, similar results were obtained with antisera S-V and Slack's group D and these two fractions.

Serological comparison of fractions P-4-3, P-3-3, and P-2-DEAE. Fractions P-4-3 and P-3-3, in a concentration of 1 mg/ml, were alternated in the six outer wells of a seven-well Ouchterlony plate, and antiserum RA I-4 was placed in the center well. A continuous, hexagon-shaped precipitin line without spurs was produced (Fig. 9). Similar results were obtained when antisera S-V and Slack's group D were substituted for RA I-4.

In additional seven-well plates, fractions P-4-3 (in concentrations of 1 and 5 mg/ml), P-3-3 (1 mg/ml), and P-2-DEAE (5 mg/ml) were alternated in the six outer wells, and antiserum was placed in the central wells. Continuous hexagon-shaped precipitin lines were obtained with both RA I-4 and Slack's group D antisera. Thus, even though these three antigenic fractions were tested simultaneously in the same plates at various concentrations, reactions of apparent identity were observed. Subsequent tests with antisera diluted 1:3 failed to alter the precipitin pattern described.

In spite of the close antigenic relationship demonstrated between fractions P-4-3 and P-3-3 and *A. bovis* antisera by agar double diffusion, the physical properties and chemical composition are significantly different (Table 2). Even after further purification of these fractions to produce fractions P-3-3-2 and P-4-3-DEAE, no differences in their antigenic relationship were demonstrated, but their properties and chemical composition are even more disparate (Table 3).

The differences in chemical composition and physical properties of the fractions (Table 3) may indicate that some of the mannose either is not added to, or is easily split off from, a polymer of high nitrogen content during growth of *A. bovis* in broth culture. This polymer of high nitrogen content might also contain mannose in a more stable linkage.

Although it was demonstrated that mannose was present by paper chromatography and color reactions, the nature of the compound containing the nitrogen was not identified. The determinants of specificity of the antigen may be associated with the nitrogen derivative, the mannose, or both.

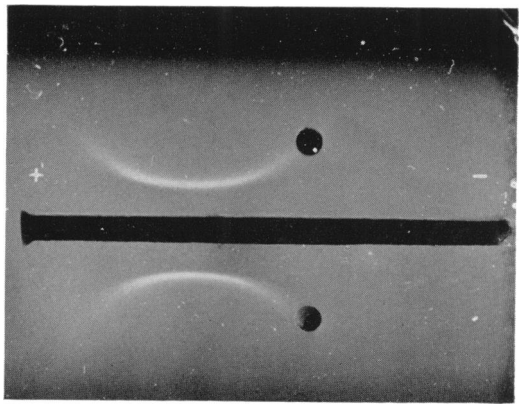

FIG. 8. *Immunoelectrophoretic pattern of Actinomyces bovis antigen P-4-3-DEAE with antiserum RA I-4.*

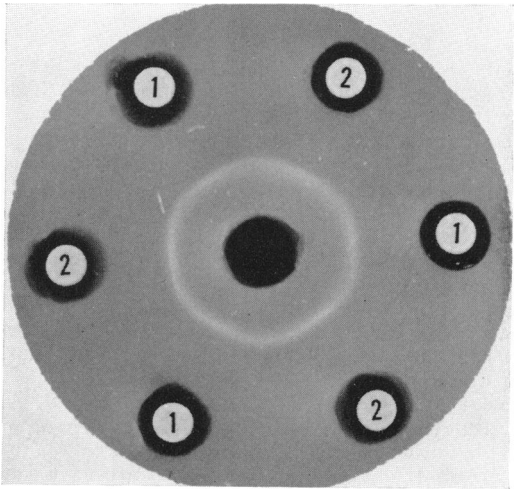

FIG. 9. *Precipitin reactions of Actinomyces bovis fractions P-4-3 and P-3-3 alternated in the outer wells with RA I-4 antiserum in the central well. Fraction P-4-3 in wells 1, and fraction P-3-3 in wells 2.*

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LITERATURE CITED

ABDEL-AKHER, M., AND F. SMITH. 1951. The detection of carbohydrate esters and lactones after separation by paper chromatography. J. Amer. Chem. Soc. **73**:5859–5860.

ANTWEILER, H. J. 1949. Quantitative microelectrophoresis. Kolloid-Z. **115**:130–137.

AOKI, M. 1936. Weitere agglutinatorische Untersuchung der Aktinomyzeten. Z. Immunitaetsforsch. **87**:196–199.

BERMAN, E. R. 1962. Isolation of bovine vitreous hyaluronic acid on diethylaminoethyl-Sephadex. Biochim. Biophys. Acta **58**:120–122.

CHARGAFF, D., C. LEVINE, AND C. GREEN. 1948. Techniques for the demonstration by chromatography of nitrogenous lipide constituents, sulfur containing amino acids, and reducing sugars. J. Biol. Chem. **175**:67–71.

COLEBROOK, L. 1921. A report upon 25 cases of actinomycosis with especial reference to vaccine therapy. Lancet **200**:893–899.

CUMMINS, C. S., AND H. HARRIS. 1958. Studies on the cell-wall composition and taxonomy of *Actinomycetales* and related groups. J. Gen. Microbiol. **18**:173–189.

DISCHE, Z. 1955. New color reactions for determinations of sugars in polysaccharides. Methods Biochem. Anal. **2**:327–355.

DRYER, R. L., A. R. TAMMES, AND J. I. ROUTH. 1957. The determination of phosphorus and phosphatase with *N*-phenyl-*p*-phenylenediamine. J. Biol. Chem. **225**:177–183.

DUBOIS, M., K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–355.

ERICKSON, D. 1940. Pathogenic anaerobic organisms of the *Actinomyces* group. Med. Res. Council Spec. Rep. Ser. 240.

GEORG, L. K., G. W. ROBERTSTAD, AND S. A. BRINKMAN. 1964. Identification of species of *Actinomyces*. J. Bacteriol. **88**:477–490.

GOYAL, R. K. 1938. A serological study of the *Actinomyces*. Indian J. Med. Res. **25**:843–846.

HEIDELBERGER, M., AND F. CORDOBA. 1956. Cross-reactions of anti-typhoid and antiparatyphoid B horse sera with various polysaccharides. J. Exp. Med. **104**:375–382.

HEYNS, K., G. KIESSLING, W. LINDENBERG, H. PAULSEN, AND M. E. WEBSTER. 1959. D-Galaktosaminuronsäure (2-amino-2-desoxy-D-galakturonsäure) als Baustein des Vi-antigens. Chem. Ber. **92**:2435–2438.

HUFFMAN, G. W., P. A. REBERS, D. R. SPRIESTERSBACH, AND F. SMITH. 1955. Nature of a hemicellulose extracted from cellulose with water. Nature **175**:990.

IKAWA, M., AND C. NIEMANN. 1949. A spectrophotometric study of the behavior of carbohydrates in seventy-nine per cent sulfuric acid. J. Biol. Chem. **180**:923–931.

KABAT, E. A., AND M. M. MAYER. 1961. Experimental immunochemistry, 2nd ed. Charles C Thomas, Publisher, Springfield, Ill.

KING, S., AND E. MAYER. 1963. Gel diffusion technique in antigen-antibody reactions of *Actinomyces* species and "anaerobic diphtheroids." J. Bacteriol. **85**:186–189.

KWAPINSKI, J. B. 1960. Antigenic structure of *Actinomycetales*. IV. Chemical and antigenic structure of *Actinomyces israelii*. Pathol. Microbiol. **23**:158–172.

MAGNUSSON, H. 1928. The commonest forms of actinomycosis in domestic animals and their etiology. Acta Pathol. Microbiol. Scand. **5**:170–245.

MATSON, C. F. 1962. A procedure for agar gel electrophoresis. Am. J. Clin. Pathol. **37**:143–149.

MCCREADY, R. M., AND E. A. MCCOMB. 1954. Determination of sugars on paper by a reflectance method. Anal. Chem. **26**:1645–1647.

PARTRIDGE, S. M. 1948. Filter-paper partition chromatography of sugars. I. General description and application of the qualitative analysis of sugars in apple juice, egg white and fetal blood of sheep. Biochem. J. **42**:238–250.

REBERS, P. A., AND M. HEIDELBERGER. 1959. The specific polysaccharide of type VI *Pneumococcus*. I. Preparation, properties and reactions. J. Amer. Chem. Soc. **81**:2415–2419.

SCHACHMAN, H. K. 1957. Ultracentrifugation, diffusion, and viscometry, p. 32–103. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 4. Academic Press, Inc., New York.

SCHMIDT, N. J., AND E. H. LENNETTE. 1955. A complement fixation test for poliomyelitis. J. Exp. Med. **102**:133–150.

SLACK, J. M., R. G. SPEARS, W. G. SNODGRASS, AND R. J. KUCHLER. 1955. Studies with microaerophilic actinomycetes. II. Serological groups as determined by the reciprocal agglutinin adsorption technique. J. Bacteriol. **70**:400–404.

SLACK, J. M., A. WINGER, AND D. W. MOORE, JR. 1961. Serological grouping of *Actinomyces* by means of fluorescent antibodies. J. Bacteriol. **82**:54–65.

TREVELYAN, W. E., D. P. PROCTER, AND J. S. HARRISON. 1950. Detection of sugars on paper chromatograms. Nature **166**:444.

WARREN, L. 1959. The thiobarbituric acid assay of sialic acids. J. Biol. Chem. **234**:1971–1975.

WEIGEL, W. W. 1960. The fractionation and reactivity of test antigens from broth cultures of *Actinomyces bovis*. M.A. Thesis, State University of South Dakota, Vermillion.

WEISSBACH, A., AND J. HURWITZ. 1959. The formation of 2-keto-3-deoxyheptonic acid in extracts of *Escherichia coli* B. I. Identification. J. Biol. Chem. **234**:705–709.

WESTPHAL, O., O. LÜDERITZ, AND F. BISTER. 1952. Über die Extraktion von Backterien mit Phenol/Wasser. Z. Naturforsch. **7b**:148–155.

WOLFF, M., AND J. ISRAEL. 1891. Ueber Reincultur des *Aktinomyces* seine Uebertragbarkeit auf Thiere. Virchows Arch. Pathol. Anat. Physiol. Klin. Med. **126**:11–59.