JOURNAL OF BACTERIOLOGY, Jan., 1967, p. 7-14
Vol. 93, No. 1

Isolation from *Pasteurella multocida* of a Lipopolysaccharide Antigen with Immunizing and Toxic Properties

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Received for publication 22 August 1966

ABSTRACT

A heat-stable, particulate, lipopolysaccharide-protein antigenic complex has been isolated from a virulent, encapsulated strain of *Pasteurella multocida* by extraction with cold, formalinized saline, and centrifugation at 105,000 × *g*. The original bacterial culture had been obtained from a bison that died of hemorrhagic septicemia, an infectious disease of cattle and buffalo. Injection of fractional milligram amounts of the antigen into mice, rabbits, and calves produced toxic reactions which frequently resulted in death of the host. The surviving animals demonstrated a high degree of immunity to challenge with live, virulent organisms. Two injections with 15 μg of the antigen produced a high degree of immunity in mice without the development of any signs of toxicity. The gross chemical composition and toxicity of the antigen were similar to those reported for endotoxins obtained by the Boivin or Westphal procedure. Although strong serological cross-reactions were obtained in Ouchterlony plates between the 105,000 × *g* antigens from the bison strain and an avian strain with antisera to these strains, these antisera agglutinated only the bacterial cells of the homologous strain. The active immunity obtained in mice by the injection of the 105,000 × *g* antigens of each strain was specific and could be correlated with the agglutination tests.

Immunizing and toxic antigens were obtained from noncapsulated avirulent mutants of two fowl cholera strains of *Pasteurella multocida*, serotypes 1 and 3 (10), by extraction with cold, formalinized saline and centrifugation at 105,000 × *g* (8). Intravenous injection of 0.5-mg amounts of these antigens, which had many of the characteristics ascribed to endotoxins, produced signs in chickens similar to those observed in acute fowl cholera.

The present work has demonstrated that similar antigens can be extracted by this method from a virulent, capsulated strain of *P. multocida*, serotype 2, which causes hemorrhagic septicemia in buffalo and cattle.

MATERIALS AND METHODS

Organisms. P. multocida strain M-1404 was used for the production of antigen, and as the challenge organism in studies on active immunity. The original culture as reported by Stein et al. (14) was isolated by L. T. Giltner in 1922 from an acute case of hemorrhagic septicemia in a buffalo in the Yellowstone National Park. The culture was maintained by serial passage in nutrient broth every 2 weeks with frequent passage through horses and mules. Since 1939, the culture has been stored in a lyophilized state at 5 C.

The same serotype was isolated in 1965 from a bison that died of hemorrhagic septicemia at the National Bison Range, Morise, Mont. (K. L. Heddleston, K. R. Rhoades, and P. A. Rebers, Am. J. Vet. Res., *in press*). A fowl cholera strain, X-73 (ATCC 11039), was used to produce antigen for studies in serological specificity and for cross-challenge experiments.

Preparation of antigen. A Tryptose Broth (Difco) suspension of the organisms was used to inoculate 150 ml of agar medium in each of 48 Roux bottles. The bottles were then incubated for 24 hr at 37 C. Dextrose Starch Agar (Difco), which was previously used for the production of antigen, was modified to reduce contamination of the particulate antigens by medium components. The gelatin and starch of the original formulation were not required for good growth and were omitted. Several commercial preparations of agar were compared, and it was found that Ionagar No. 2 (Consolidated Laboratories, Chicago Heights, Ill.) gave the smallest amount of gel-like precipitate after extraction with cold saline and centrifugation of the extract at 105,000 × *g* (40,000 rev/

min) for 2 hr in a Spinco model L centrifuge with a no. 40 rotor. The modified medium contained the following ingredients per liter of distilled water: 15 g of Proteose Peptone No. 3 (Difco), 2 g of glucose, 5 g of NaCl, 3 g of Na_2HPO_4, and 20 g of Ionagar No. 2. The ingredients were dissolved by heating in a boiling-water bath, the solution was cooled to 60 C, and the *p*H was adjusted to 7.4. It was then dispensed into the Roux bottles and autoclaved for 20 min.

After 24 hr of incubation at 37 C, the cells were washed from the agar of each bottle with 10 to 12 ml of 0.85% NaCl solution containing 0.3% Formalin. The suspension of cells was mixed on a rotary shaker (170 rev/min) for 24 to 48 hr at 4 C. The cells were then removed by centrifugation for 30 min at 13,000 × *g*, and the supernatant fluid was clarified by filtration through a 400-mesh nylon cloth (Tobler, Ernst, and Traber, Inc., New York, N.Y.). The filtrate was centrifuged at 105,000 × *g*. The supernatant fluid was decanted, and the small gel-like pellets were resuspended in formalinized saline (0.85% NaCl, 0.1% sodium acetate, 0.1% Formalin, *p*H 7.2) and allowed to stand overnight at 4 C; the suspension was recentrifuged at 105,000 × *g*. Sodium acetate was added to the formalinized saline to raise the *p*H to about 7, since it was observed that partial precipitation of the antigen occurred at *p*H 4.5. After 3 washes with formalinized saline, the pellets were suspended in 20 ml of distilled water solution of 0.1% Formalin and 0.1% sodium acetate, *p*H 7.2, by means of a syringe and a no. 20 cannula. The suspension was allowed to stand at 4 C at least 3 days, and was centrifuged at 12,000 × *g* for 30 min to remove a small amount of precipitate, which was discarded. After dialysis and lyophilization, the solids content in the slightly opalescent supernatant fluid was found to be 2 to 2.5 mg/ml. This suspension was designated M-1404 antigen in the following studies.

Experimental animals. Holstein-Friesian calves (13 to 18 weeks old), young, mature, white New Zealand rabbits, and female Swiss-Webster mice (18 to 28 g) were used where indicated.

Toxicity and immunity. Calf 1 was inoculated intravenously (iv) with 1.8 mg of antigen, followed in 1 hr and 3.5 hr with similar inoculations. Calf 2 was inoculated iv with 1.4 mg of antigen and 1.5 hr later with 1.8 mg. Calf 3 was inoculated iv with 0.9 mg of antigen. A fourth calf not inoculated with the antigen was used as a control. At 2 weeks after injection of the antigen, blood samples were taken from calves 2, 3, and 4, and then their immunity was challenged with an intramuscular injection in the neck of approximately 5×10^9 viable cells of a 24-hr culture of M-1404. Rectal temperatures were recorded twice a day for 1 week before inoculation with antigen and 4 weeks after exposure to live organisms.

Sera of calves 2, 3, and 4 from bleedings taken just before challenge of the calves with live organisms were evaluated for their ability to immunize mice passively. Mice were inoculated intraperitoneally (ip) with 0.1 ml of serum and challenged the next day with an ip inoculation of approximately 800 organisms. A total of 10 mice were used with each antiserum, and 10 noninoculated mice were used as controls. Surviving mice were observed for at least 7 days.

The toxicity of the antigen was evaluated in rabbits and mice after iv and ip inoculation, respectively. Active immunity of rabbits surviving toxicity tests was determined by intramuscular challenge with approximately 1,700 organisms, whereas that of the mice was determined by ip challenge with approximately 1,000 organisms.

Assay methods. Nitrogen was determined with a micro-Kjeldahl method (9), total phosphorus with the p-semidine method (4), total carbohydrate by the phenol-sulfuric acid method with glucose as the standard (5), heptose with the cysteine-sulfuric acid method of Dische (3) and with Osborn's (11) modification of the cysteine-sulfuric acid method. The total amount of fatty acids combined as esters and amides was determined with Haskins' (6) modification of the hydroxylamine-ferric perchlorate reaction. All spectral measurements were obtained with a Beckman model DK-2a recording spectrophotometer.

Ouchterlony plates were prepared with 0.6% Ionagar No. 2 in 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.2) and 0.1 M NaCl, incubated at 22 C, and observed daily for 1 week. The holes were punched by use of cutters 4 mm in diameter, with center to center spacings of 1 cm. The antisera used in these plates were prepared as follows.

Rabbit antisera to the M-1404 antigen were prepared by biweekly intravenous inoculation with 20 μg each for 1 month, followed with biweekly injections of 60 μg each for 2 months. After that time the rabbits were bled by heart puncture.

Rabbit antisera to the X-73 antigen were prepared by two intravenous injections of 16 μg given over a 2-week period, followed by bleeding 1 month after the first injection.

Plate agglutination tests were carried out as previously described (7).

RESULTS

Immunogenic and toxic properties of the M-1404 antigen. When injected in milligram amounts, the antigen was both toxic and immunogenic for calves, rabbits, and mice. The earliest sign of toxicity in calves given iv injections of the antigen was rapid, shallow breathing, which occurred within 5 to 15 min. Depression, increased salivation, and lacrimation occurred a short time later. Approximately 30 min after the initial inoculation, diarrhea was observed; at first it was mucoid, but later became watery. The rate of salivation continued to increase, and a mucoid nasal discharge became apparent (Fig. 1). The degree of depression progressed to the point where the animals were recumbent and could not be forced to rise. In calf 1, depression continued through a comatose stage and terminated in death 4.5 hr after the first injection. Repeated determinations of rectal temperature did not

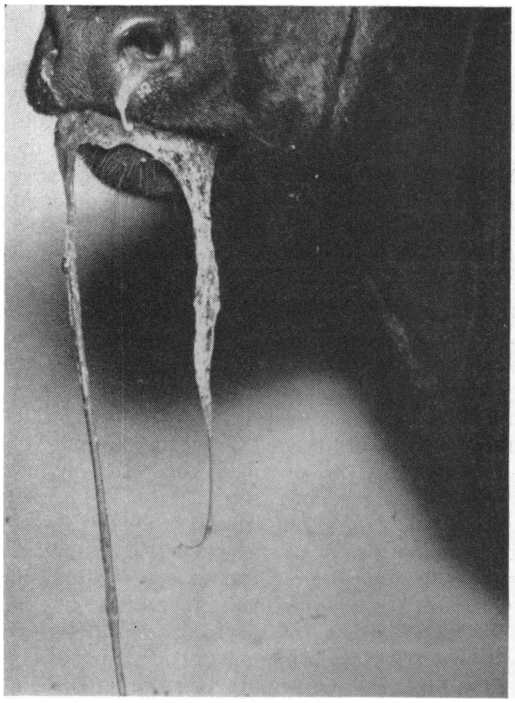

FIG. 1. *A. 13-week-old calf 1 hr after injection of 1.8 mg of the M-1404 antigen.*

reveal a variation of more than 1 F from the temperature prior to inoculation. Calves 2 and 3, which received progressively smaller amounts of the antigen, showed similar signs, but were completely recovered in 48 hr. The immunity of calves 2 and 3, along with that of the control (calf 4), was challenged 2 weeks later with viable *P. multocida* M-1404. No signs of infection appeared in either calf 2 or 3, but the control died in 18 hr of acute hemorrhagic septicemia. The macroscopic and microscopic lesions of calves 1 and 4 will be reported in a separate paper (K. R. Rhoades, K. L. Heddleston, and P. A. Rebers, Can. J. Comp. Med. Vet. Sci., *in press*).

Sera from calves inoculated with M-1404 antigen were evaluated for their ability to protect mice passively against challenge with live organisms from the homologous strain, as well as against the apparently identical Insein strain P-1256 of Asia, and the related but not identical African strain, P-1234 (K. L. Heddleston, K. R. Rhoades, P. A. Rebers, Am. J. Vet. Res., *in press*). The serum from calf 2 which received 3.2 mg of antigen protected mice against all three strains, whereas serum from calf 3 which received only 0.9 mg of antigen protected mice only against strains M-1404 and P-1256. The results are summarized in Table 1.

To determine the toxicity and immunogenicity of the M-1404 antigen in mice, 14- to 350-μg amounts of the antigen were injected ip. No toxic effects were observed with 14 to 30 μg, but, with 250 to 350 μg, depression, diarrhea, ruffled hair coats, and lacrimation were observed 1 to 2 hr after injection. All deaths from the toxicity of the antigen occurred within 1 to 2 days. The immunity of the surviving mice was challenged with a homologous culture 3 to 4 weeks after injection of the antigen. The data describing the toxic and immunizing properties of the antigen in mice are summarized in Table 2. The highest degree of immunity was obtained with two injections of 15 μg of antigen given over a 10-day interval. Of these mice, 100% were protected against a challenge dose which killed 100% of the controls in 1 to 2 days. Neither treatment of the antigen with 5% Formalin, nor the heating for 10 min at 100 C appeared to alter its toxicity or immunogenicity to any significant extent.

The development of toxic signs in rabbits after intravenous injection of the M-1404 antigen was more variable than that observed for mice. Occasionally, as little as 15 μg was sufficient to cause death, whereas, in other cases, as much as 300 μg was tolerated. The toxic signs consisted of diarrhea, depression, dyspnea, and a febrile response which reached a maximum 2 to 4 hr after injection, and subsided the next day. The temperature-response curves of four rabbits injected with 300-μg amounts of the antigen were compared with those of rabbits injected with the formalinized water used as the suspending medium (Fig. 2).

Four more rabbits were inoculated with 500 μg

TABLE 1. *Passive immunization of mice with calf antisera to the M-1404 antigen*

Serum	Challenge strain		
	M-1404	P-1234	P-1256
Calf 2[a]	3/20[b]	2/10	0/10
Calf 3[c]	2/20	10/10	0/10
Calf 4[d]	10/10	—	—
No serum	10/10	10/10	10/10

[a] Calf 2 was inoculated with 3.2 mg of the M-1404 antigen, and bled 2 weeks later.

[b] Number of deaths after 7 days per number challenged.

[c] Calf 3 was injected with 0.9 mg of antigen, and bled 2 weeks later.

[d] Calf 4 was a normal calf which was used as a control.

TABLE 2. *Toxic and immunogenic properties of the M-1404 antigen in mice*

Amt injected	Treatment of antigen	Deaths/no. inoculated	Deaths/no. inoculated[a] with live organisms
μg			
14		0/30	18/29
14	Heated[b]	0/20	14/20
15, 15[c]		0/10	0/10
15, 15[c]	Heated[b]	0/10	0/10
30		0/20	9/19
300		12/39	7/26
300	5% Formalin[d]	3/10	1/7
300	Heated[b]	0/10	3/10
356		3/10	7/7
356	Heated[b]	6/10	4/4
Controls		—	20/20

[a] A challenge dose of about 800 organisms was given 3 weeks after the last injection.

[b] Heated 10 min in a boiling-water bath.

[c] Second injection given 10 days after the first.

[d] Treated with 5% Formalin for 24 hr at 22 C, then dialyzed against saline containing 0.1% Formalin.

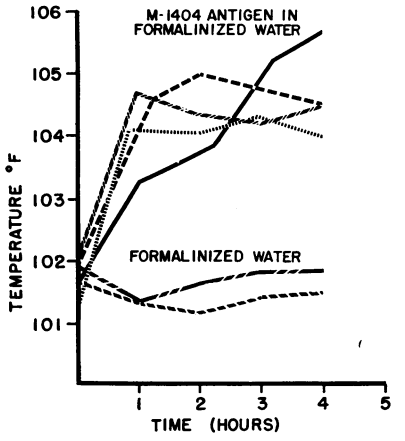

FIG. 2. *Upper curves show temperature response of rabbits to the injection of 300-μg amounts of the M-1404 antigen in formalinized, distilled water. Lower curves show temperature response of rabbits to formalinized, distilled water.*

of the antigen. An average increase in rectal temperature of 3.2 F was obtained 4 hr after injection. Two rabbits died in less than 24 hr; the survivors were challenged 11 days later with an injection of 500 μg, but no toxic effects were observed this time and the temperatures remained normal. The immunity of the inoculated rabbits was determined by challenge with live bacteria 3 to 4 weeks later. Eight of nine rabbits survived a challenge dose which killed all four controls. The results are summarized in Table 3.

Cross-reactions and cross-protection with strain X-73. The serological specificity of the M-1404 antigen was evaluated by double-diffusion techniques in agar gel against rabbit antisera to the M-1404 antigen and to the X-73 105,000 × *g* antigen (8). Antisera from at least six different rabbits with four separate bleedings of each rabbit were evaluated with the homologous and the heterologous antigens. Samples of the most specific as well as the most cross-reactive antisera were selected to illustrate differences in specificity that were observed with different rabbits. Photographs of the Ouchterlony plates are shown in Fig. 3. Those shown in Fig. 3b, d, and f illustrate the relatively high degree of specificity that was obtained with relatively few rabbit antisera, whereas those shown in Fig. 3a, c, and e show the strong cross-reactions that were obtained with most of the antisera. It is obvious that both antigen preparations are mixtures in which one major component predominates.

A higher degree of specificity was observed with the plate agglutination test (7). Five of six rabbit antisera, and each of two calf antisera to the M-1404 antigen, agglutinated M-1404 cells; however, none of these antisera agglutinated X-73 cells.

The rather strong cross-reactions obtained in the Ouchterlony plates indicated that the antigens might be cross-protective, and the following experiments were designed to test this. A group of 20 mice were given two injections of 14 μg of M-1404 antigen over a 10-day period, and another

TABLE 3. *Toxic and immunizing properties of the M-1404 antigen in rabbits*

Amt injected	Deaths/no. inoculated	Deaths/no. inoculated with live organisms[a]
μg		
15	0/2	1/2
15[b]	1/2	0/1
20	0/2	—
73	1/2	—
300	0/4	0/4
500	2/4	—
500[c]	0/2	0/2
Controls	—	4/4

[a] Intramuscular challenge containing 1,700 bacteria given 3 to 4 weeks after injection of antigen.

[b] Antigen heated 10 min at 100 C before injection.

[c] Rabbits surviving first injection of 500 μg were given injections 11 days later.

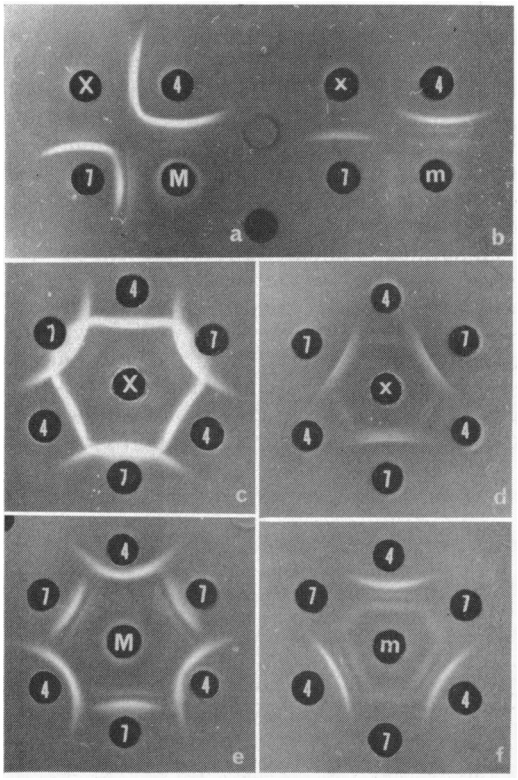

FIG. 3. *Reaction of 105,000 × g antigens from strains M-1404 and X-73 with selected rabbit antisera to these antigens: 7, X-73 antigen; 4, M-1404 antigen; X, x, antisera from different rabbits to X-73 antigen; M, m, antisera from different rabbits to M-1404 antigen.*

group of 20 were injected twice with 10-μg amounts of X-73 antigen. Both groups were challenged 1 month later after being divided so that half of each group was challenged with the homologous, and the other half with the heterologous, organism. The mice receiving the homologous challenge were protected against a dose of organisms which killed all of the controls, but cross-protection was not observed. The results are summarized in Table 4.

Chemical analysis. The M-1404 antigen was analyzed for total carbohydrate, hexose, heptose, ketodeoxyoctonic acid, total nitrogen, total phosphorus, and fatty acid esters and amides as described in Materials and Methods. The chemical analyses were within the rather wide range of values reported for various preparations of endotoxins. The data for four different lots of antigen are summarized in Table 5. The variations in composition from lot to lot provide additional evidence of the nonhomogeneity of the preparations. The cysteine-sulfuric acid reaction for

TABLE 4. *Active immunity of mice injected with 105,000 × g antigens of Pasteurella multocida to homologous and heterologous challenge*

Source of antigen injected	Strain of organism used for challenge	
	M-1404	X-73
M-1404	0/10[a]	10/10[a]
X-73	10/10	1/10
Controls	10/10	10/10

[a] Deaths per number challenged 7 days after challenge.

TABLE 5. *Chemical composition of the M-1404 antigen*

Determination	Per cent of composition in lot no.			
	1	2	3	4
Total nitrogen	5.9	4.7	5.7	—
Total carbohydrate[a]	20.5	19.0	20.4	23.5
Total phosphorus	1.7	1.0	1.4	—
Combined fatty acids[b]	—	—	12.0	10.0

[a] Determined with the phenol-sulfuric acid method with glucose as a standard.

[b] Determined according to the method of Haskins (6) with tripalmitin as the standard.

heptoses (3) with the 3-min period at 100 C resulted in a product with an absorption curve having maxima at 600 and 390 mμ and a shoulder from 500 to 550 mμ (Fig. 4). At 24 hr after the addition of cysteine, the curve obtained with the antigen in the range of 575 to 650 mμ was identical to that obtained with the galactose standard, but was easily differentiated from that of glucose, whereas that of mannose showed almost no absorption. The modified cysteine-sulfuric acid reaction (11) with the 10-min period at 100 C was used to estimate the total heptose in the presence of interfering hexoses. The absorbancy of the curves was measured 2 hr later at 505 and 545 mμ, and the heptose calculated from the formula given by Osborn ($A_{505} - A_{545} = 1.07$ per micromole of L-glycero-D-mannoheptose) was found to be 5% for lot no. 1. However, when the calculations were made with D-glycero-D-galoheptose as the standard, a figure of 10% was obtained. Until the heptose is qualitatively identified, the quantitative determinations are only approximate. The absorption curves obtained with the modified cysteine-sulfuric acid reaction for heptoses were obtained 2, 24, and 72 hr after the addition of cysteine. Those obtained 24 hr after

the addition of cysteine are shown in Fig. 5. The method eliminates interference by hexoses. Definite peaks at 500 mμ are seen with both the standard of D-glycero-D-galoheptose and the M-1404 antigen, whereas galactose shows only a slight nonspecific absorption over the range of 400 to 700 mμ, which is in definite contrast to the curve shown in Fig. 4.

The possible presence of 2-keto-3-deoxyoctonic acid in the M-1404 antigen was determined with the periodate-thiobarbituric acid reaction of Aminoff (1). A standard solution of 2-keto-3-deoxyoctonic acid was prepared by the alkaline hydrolysis of penta-acetyl-3-deoxyoctulosonate methyl ester according to the procedure of Heath (*Methods in Enzymology, in press*). The amount of color produced in the reaction from the M-1404 antigen was a function of the conditions of hydrolysis. For comparison, 1, 0.4, 0.1, and 0.01 N HCl solutions of the antigen were heated at 100 C for 1 hr, and the standard solution was treated in the same manner. The maximal yield of color was produced from the antigen solution after hydrolysis for 1 hr with the 0.01 N HCl. The yield of color produced from the standard solution decreased with heating, probably as a result of lactonization, and, after 1 hr at 100 C with 0.01 N HCl, only 50% as much color was obtained as with the unheated, standard solution. The absorption curves of the products obtained with the antigen as well as those from the standard are shown in Fig. 6. The absorption peak of the antigen at 548 mμ, which corresponds to that obtained with the standard, is presumptive evidence

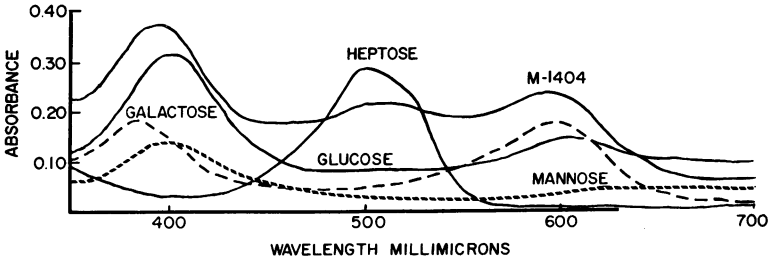

FIG. 4. *Absorption curves of the products of the cysteine-sulfuric acid reaction, 3 min at 100 C, 24 hr after the addition of cysteine; D-galactose, 50 μg; D-glucose, 50 μg; D-mannose, 50 μg; D-glycero-D-galoheptose, 55 μg; and M-1404 antigen, 356 μg.*

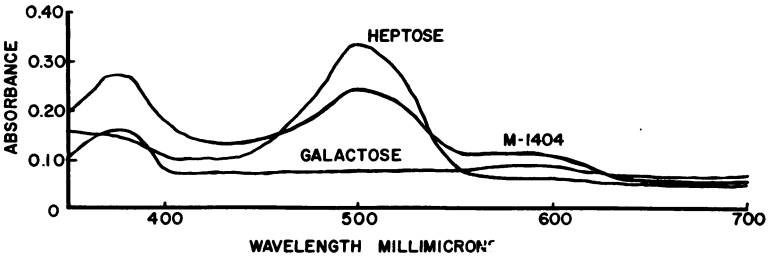

FIG. 5. *Absorption curves of the products of the cysteine-sulfuric acid reaction, 10 min at 100 C, 24 hr after the addition of cysteine; D-galactose, 50 μg; D-glycero-D-galoheptose, 50 μg; and M-1404 antigen, 356 μg.*

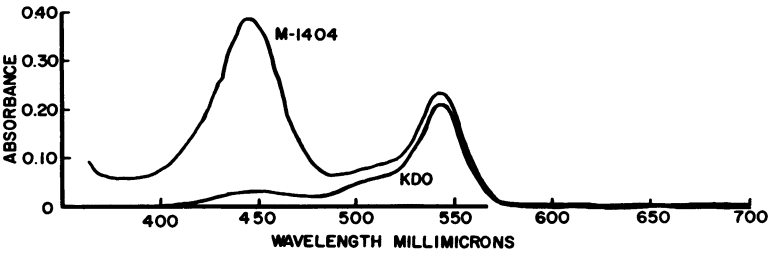

FIG. 6. *Absorption curves of the products of the periodate-thiobarbituric acid reaction. KDO, 8.3 μg of 2-keto-3-3-deoxyoctonic acid; M-1404 antigen, 350 μg; conditions of hydrolysis, 1 hr at 100 C with 0.1 N HCl.*

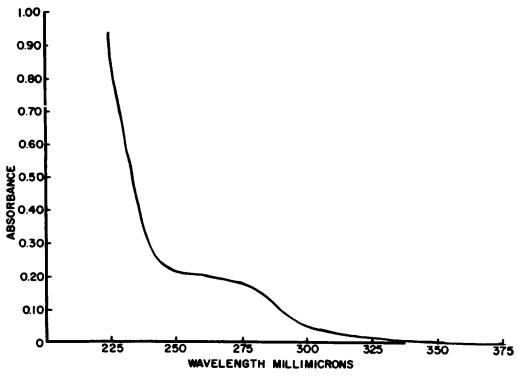

FIG. 7. *Ultraviolet absorption curve of the M-1404 antigen in 0.1% sodium acetate at pH 7.2.*

of the presence of approximately 2% 2-keto-3-deoxyoctonic acid in the M-1404 antigen.

The ultraviolet absorption curve of the M-1404 antigen in 0.1% sodium acetate at *p*H 7.2 is shown in Fig. 7. A definite plateau was obtained from 250 to 275 mμ, which is indicative of the presence of protein or peptide. No alteration of the curve was seen after the antigen solution was heated for 10 min at 100 C.

DISCUSSION

The M-1404 antigen from a capsulated virulent strain of *P. multocida*, which can cause hemorrhagic septicemia in cattle, had many properties in common with a similar preparation obtained from a noncapsulated avirulent strain of avian origin, X-73 (8). The intravenous injection of milligram amounts of the M-1404 antigen in calves, or of the X-73 antigen in chickens, resulted in the appearance of toxic signs that closely resembled those which appear after the challenge of the susceptible host with the virulent organisms. After recovery from the toxic effects of a sublethal amount of the antigens, a high degree of active immunity to challenge with live organisms of the homologous strain was demonstrated previously with chickens, mice, and rabbits, and in this investigation with calves, mice, and rabbits. Inoculation of mice with small nontoxic amounts of the antigen also resulted in the development of a high degree of active immunity. Mice have been actively immunized with lipopolysaccharide-protein complexes from other strains of *P. multocida* by Pirosky (13), who used the Boivin procedure for the extraction of the antigens. However, the lipopolysaccharide antigen obtained by Bain and Knox (2) with the Westphal procedure from a strain apparently identical to the M-1404 (K. L. Heddleston, K. R. Rhoades, and P. A. Rebers, Am. J. Vet. Res., *in press*) failed to immunize mice actively, even though precipitins could be detected in rabbit serum after injection. The antigen obtained by Perreau and Petit (12) with the Westphal procedure also failed to induce active immunity in mice. It is possible that treatment with phenol of an antigenic complex composed of lipid, protein, and polysaccharide results in the dissociation of the components, so that the lipopolysaccharides remaining in the aqueous phase are no longer immunogenic for mice. Treatment of water solutions of the M-1404 antigen with phenol resulted in the formation of a large amount of a stable emulsion; thus, the study of the effect of phenol on the antigen was not undertaken at this time.

The ultraviolet absorption curve of the aqueous solution of the antigen suggested that protein might be present, but no change in the absorption curve occurred after heating for 10 min at 100 C. Furthermore, heating had no effect on the toxic or immunogenic properties of the antigen or on the patterns obtained in Ouchterlony plates.

The double-diffusion patterns in agar with the M-1404 and X-73 systems indicate that both are mixtures which contain a major and one or more minor components. The high degree of cross-reaction obtained with most of the antisera could be the result of antibodies directed against a common component which is present in both preparations. However, the specific reactions obtained with the proper choice of antisera indicate that separate and specific antigens may be present in each mixture, and that these specific antigens, at least with mice, include those capable of inducing specific active immunity.

The possible presence of specific antigens on the cell surface was suggested by the specific agglutinations obtained with the homologous system in the plate agglutination test, which correlated with the results obtained by the active immunization of mice.

The M-1404 antigens resemble endotoxin preparations in chemical composition, heat stability, and toxicity, but they are better antigens from the standpoint of inducing active immunity than the lipopolysaccharides isolated from similar strains of *P. multocida* by the Westphal procedure.

The active immunity induced by the M-1404 antigen could result from the presence of traces of a nontoxic, specific, capsular antigen rather than from the lipopolysaccharide complex. The related lipopolysaccharide preparation from a noncapsulated strain, X-73, also induced a high degree of active immunity, and, although the presence of specific capsular antigens in this

preparation is less likely, it is still possible. The determination of the immunizing capacity of the lipopolysaccharide component(s) in these preparations cannot be finally made until further studies on their purification have been carried out.

ACKNOWLEDGMENTS

We are grateful to Edward C. Heath for a crystalline sample of penta-acetyl-3-deoxyoctulosonate methyl ester, and for helpful discussions concerning its properties. We also acknowledge the high quality of technical assistance of John DeLance in the laboratory and of Ralph Glazier in preparing the illustrations.

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