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THE EFFECT OF FORMALIN-KILLING OF *Pasteurella multocida* ON THE ANTIGENICITY AND EXTRACTABILITY OF ITS LIPOPOLYSACCHARIDE

PAUL A. REBERS AND RICHARD B. RIMLER
National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, P.O. Box 70, Ames, Iowa 50010 (U.S.A.)*

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ABSTRACT

The extraction of lipopolysaccharides (LPS) from formalin-killed (FK) *Pasteurella multocida* strain X-73 and from cells not exposed to formalin (NF) were compared by the Westphal and phenol–chloroform–petroleum ether (PCP) extraction procedures. The LPS was determined by: (*1*) serologic analyses with antiserum specific for LPS; (*2*) analyses for toxicity; and (*3*) chemical analyses for components expected to be in LPS (such as hexoses, heptoses, amino sugars, 3-deoxyoctulosonic acid, and fatty acids). Strain X-73, the strain most virulent for chickens, was markedly affected by formalin killing. Unlike many strains, which readily yield LPS into the aqueous phase when extracted with phenol at 68° by the Westphal procedure, strain X-73 did so only with FK and not with NF cells. With the NF cells, LPS was extracted by EDTA from the precipitate obtained during the Westphal procedure. With the PCP procedure, LPS was extracted readily from NF cells, but not from FK cells. The change in extractability of LPS as a result of formalin-killing was the same for both the encapsulated form of X-73 and a nonencapsulated variant derived from it. Although formalin-killing affected the extractability of LPS, no antigenic differences could be detected by immunodiffusion. However, the chick-embryo toxicity of LPS extracted from NF cells was greater than that of LPS from FK cells.

INTRODUCTION

In our initial studies of the lipopolysaccharide (LPS) of *Pasteurella multocida*, we have prepared potent vaccines by killing the bacteria with formalin. In preparing these vaccines, we added formaldehyde to the bacterial culture, incubated the mixture, and then checked it for sterility and the presence of excess formaldehyde. As the vaccine induced immunity against the test organism, it was considered satisfactory.

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*No endorsements are implied herein.

However, chemical changes of the antigens resulting from reactions with formaldehyde are possible, and some effects of formalin-killing on the extraction and purification of bacterial components have been reported. Formalin-killing decreased the amount of RNA extracted from *Salmonella* bacteria by the Westphal (phenol–water) procedure without affecting the amount of LPS extracted[1]. The LPS extracted by phenol–water from formalin-killed *Salmonella typhimurium* contained 3% of ribose, whereas the LPS extracted from acetone-killed cells contained 19% of ribose[2]. Knox and Bain[3] reported that isoelectric fractional precipitation of saline extracts from freeze-dried live *P. multocida* was successful, but that the isoelectric fractionation failed to work with extracts from formalin-killed cells. The failure was attributed to cross-linkages of the fractions, induced by reaction with formalin.

The possible chemical changes in LPS as a result of formalin-killing are of particular interest to us. The LPS of *P. multocida* form the basis of the gel-diffusion-precipitin test[4,5], which is widely used for typing isolates; this test is one of the few that give good correlation between serological type and immunological type as determined by vaccination and challenge. The typing antigen is prepared by heating a bacterial suspension with formalinized saline at 100°, thus inactivating protein antigens and increasing the solubility of a complex containing LPS. The typing antisera may be prepared by inoculating chickens with formalin-killed *P. multocida* cells, but recent research has shown that a more-specific typing antiserum may be prepared by inoculating chickens with purified[6] LPS. Chickens are used, rather than mice or rabbits, because they exhibit[7,8] a much stronger antibody response to *P. multocida* LPS.

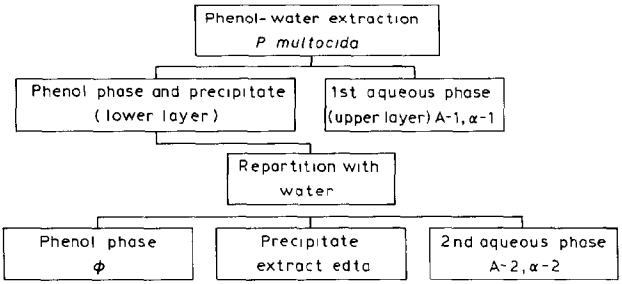
We investigated the effect of formalin-killing on the extractability, antigenic properties, toxicity, and chemical composition of *P. multocida* LPS. Two methods of extraction were compared: the phenol–water procedure of Westphal[1,9], which generally works well with both smooth- and rough-type LPS, and the phenol–chloroform–petroleum ether (PCP)[10] procedure, which generally works only with rough-type LPS. As LPS was not found in the aqueous or phenol layers of the Westphal extracts of the NF cells, the precipitate produced during the Westphal procedure was extracted with ethylenedinitrilo(tetraacetic acid) (EDTA). The extractions were performed with a virulent, encapsulated *P. multocida* and a nonencapsulated variant, because the chemical composition and extractability of the LPS might change with capsular type. The amount of LPS in the fractions was evaluated by serology with specific LPS antisera, by the chicken-embryo toxicity test, by the *Limulus* amebocyte lysate test, and by chemical analyses for components expected to be in lipopolysaccharides.

MATERIALS AND METHODS

Bacteria. — Two colonial forms of *P. multocida* (strain X73, ATCC 11039, Heddleston's serotype 1) were used: the iridescent, designated X73I and the blue,

designated[11] X73B. Strain X73I (also called X73F previously) was originally isolated from a chicken; it is encapsulated and highly virulent for chickens, rabbits, and mice and develops an iridescent colonial form when cultured on agar. The blue colonial form, X73B, is a nonencapsulated, much less virulent, variant of the iridescent form.

Culture, formalin-killing, and Westphal extraction. — *P. multocida* variant X73B was cultured in 35 L of modified Adams Roe Broth[12] (without thioglycolate) and with enzymically digested casein. The mixture was incubated for 6 h at 37° (end of log-phase growth) in a New Brunswick Labroferm (Edison, NJ) fermentor. The cell suspension was divided into two equal portions. The cells in one half were killed by the addition of 0.3 mL of 37% formaldehyde (formalin) per 100 mL of culture at pH 6.2–6.4. After 48 h at 4°, no viable cells were detected in the mixture and these cells were designated formalin-killed (FK). Under these conditions, an excess of formaldehyde is present and may be detected by the Schiff reagent. No formalin was added to the other half of the culture (NF cells). Each suspension was concentrated to a volume of 1 L with a Millipore Pellicon Cassette System (Bedford, MA) containing GSWP membranes. The NF and FK concentrates were centrifuged for 1 h at 13,000g and the cells were washed with saline, then with distilled water. The washed cell pellet (41 g wet weight) was suspended in 190 mL of distilled water and heated to 68°; and extracted by the Westphal procedure[1]. Fractions were collected and labeled as shown in Scheme 1; when FK cells were extracted, the aqueous layers were labeled A1 and A2. When NF cells were extracted, the aqueous layers were labeled $\alpha 1$ and $\alpha 2$. The first aqueous layer was siphoned off, and the phenol layer (ϕ) and precipitate were extracted a second time with water at 68°. The second aqueous layer and the phenol layer were removed (Scheme 1). The residual precipitate was extracted with EDTA as described in the next section. In one experiment, a third extraction of the phenol layer was performed and the antigens were designated A3 (from FK cells).



Scheme 1. Westphal extraction procedure.

Because, in preliminary studies, *P. multocida* strain X73I cultured in Adams Roe medium tended to obstruct the Millipore Pellicon Cassette System during concentration, we cultured it on a D-glucose–starch–agar medium[11] (Difco, Detroit)

for 24 h at 37° and one-half of the culture was harvested with a small volume of sterile saline, and the other half was harvested with saline containing 0.3 mL formalin per 100 mL. The cells were washed and extracted by the Westphal procedure as described with the X-73B variant.

EDTA extractions of precipitate from NF cells. — In the Westphal extraction of FK cells, only a small amount of precipitate was obtained and this was discarded. However, large amounts of a stringy precipitate were obtained with the NF cells. The precipitate was stirred with 200 mL of 0.04M EDTA (pH 8) containing 0.03M sodium azide for 5 h at 22°. The precipitate became granular after stirring. The mixture was centrifuged, and the pellet re-extracted with fresh EDTA–azide for 4 h at 60°. After centrifugation, the supernatant solutions from the 22 and 60° extractions were dialyzed exhaustively against distilled water. After the extracts had been tested for RNA and DNA, they were treated with RNase and DNase[7] in 0.1M glycine buffer (pH 8) with 0.01M Mg^{2+}; two 10-mL portions of the digest were centrifuged for 2 h at 105,000*g*. The upper 9 mL of the supernatant solutions were carefully aspirated and discarded, as they were inactive in the immunodiffusion test. The pellets and lower 1 mL of the supernatant solutions were washed three times by resuspension and recentrifugation at 105,000*g* for 2 h. After the final centrifugation, the lower 1 mL of solution and the pellet were collected separately and analyzed as described later.

PCP extraction. — *P. multocida* variant X73B and strain X73I were cultured as previously described, and either killed with formalin, or not treated with formalin at all. The cells were collected as before, washed twice with distilled water, dried with ethanol and ether, and extracted by the PCP procedure[10].

Animals. — White Leghorn and New Hampshire roosters were obtained from the National Animal Disease Center and housed as previously described[8]. Nicholas Broad Breasted turkeys were obtained from the Jerome Hatchery, Barron, WI.

Antisera. — Antisera for the general purpose of detecting *P. multocida* antigens in the extracts were prepared by inoculating chickens or turkeys intravenously with whole-cell preparations of formalin-killed *P. multocida* strain X73I as described[13].

Because purified standard *P. multocida* LPS was not available, antisera to *P. multocida* LPS were prepared by inoculating birds with three different preparations of purified LPS: (*1*) LPS extracted by the Westphal procedure from *P. multocida* X73I FK cells, and then treated with RNAse and DNAse, purified by ultracentrifugation, and fractionated in a CsCl gradient[7]; (*2*) LPS extracted by EDTA from the precipitate of the Westphal extraction of *P. multocida* X73B NF cells; and (*3*) LPS extracted by the PCP procedure from *P. multocida* X73B NF cells.

Antisera were prepared by inoculating roosters intravenously twice weekly with 1 mL of LPS solution containing 25–28 μg LPS. The sera were collected 3 weeks after the start of the inoculations. Each serum was kept separate and evaluated by immunodiffusion.

Chemical and serological analyses. — Immunodiffusion tests were performed in agarose gel with [4] 1.45M NaCl; the wells were 3 mm in diameter and 8 mm apart (center-to-center). The amounts of hexose and heptose in each fraction were determined by a modified cysteine–sulfuric acid reaction[7]. Neutral sugars were determined by hydrolysis for 48 h with 0.2M HCl at 100° followed by column chromatography with an ion-exchange resin in the lithium form[14]. Total carbohydrate was determined by the phenol–sulfuric acid reaction[15]. Amino sugars and amino acids were determined with a Beckman amino acid analyzer[7]; KDO was determined by the method of Karkhanis[16]; and DNA by the diphenylamine procedure[17]. Fatty acids were identified from the retention times of their methyl esters by gas chromatography with a Hewlett–Packard 5380 gas chromatograph equipped with a column of 3% SP 2100 DOH (Supelco, Bellefonte, PA)[18]. 2-Hydroxytetradecanoic acid was further characterized by the change in retention time obtained after treating the methyl ester with trifluoroacetic anhydride[18]. Bromination was used to test for unsaturated fatty acids[18].

Electron microscopy. — Lipopolysaccharides were suspended in water and positively stained with uranyl acetate[19]. The samples were examined with a Philips 200 electron microscope at 60 kV.

Determination of endotoxin activity. — The activity of the LPS fractions was evaluated by the Limulus amebocyte lysate test[20] and reported as the highest dilution capable of inducing gel formation.

The toxicity was determined with chick embryos by the chorioallantoic membrane procedure[21]. Four different dilutions of each LPS fraction were inoculated into groups of 10–12 eggs. The number of surviving embryos was determined after 1 day, and the LD_{50} and 95% confidence limits were calculated by the probit procedure. All dead embryos were cultured for microbial contamination, and contaminated embryos were omitted from calculations of number living versus number dead.

RESULTS

Westphal and EDTA extractions. — Each of the Westphal fractions obtained from FK or NF *P. multocida* X-73 B was initially tested by immunodiffusion[4] with LPS antisera prepared from FK cells (Table I). Fraction A2 reacted more strongly in immunodiffusion than A1, α1, or α2. When the same fractions were subsequently evaluated with antisera prepared with LPS from NF cells (Table I), fraction A2 also reacted more strongly than A1, α1, or α2. Hence, A2 had the most LPS; A1, α1, and α2 had little LPS. The EDTA extract from the NF cells contained LPS; the 60° extract contained more LPS than the 22° extract. The phenol fraction (Scheme 1) from FK or NF cells was also active, but relatively little material was obtained from the phenol layer (Table I).

The FK or NF *P. multocida*, X73I, was also extracted by the Westphal procedure, and the extracts analyzed by immunodiffusion as already described. Fraction

TABLE I

EFFECT OF FORMALIN KILLING ON EXTRACTION OF LIPOPOLYSACCHARIDES OF *Pasteurella multocida* STRAIN X73B AND X73I

Fraction[a]	*Yield*[b]	*Immunodiffusion results*			
		Antisera prepared with LPS from FK cells		*Antisera prepared with LPS from NF cells*	
		Whole[c] *cell*	*CsCl-LPS*	*EDTA-LPS*	*PCP-LPS*
LPS extracted by phenol–water method of Westphal					
Strain X73B (no capsule)					
Formalin killed					
A1	675	+	–[d]	+ or –	ND
A2	500	+++	+++	+++	+++
ϕ	13	+	+	ND	ND
No formalin					
α1	310	+ or –	–	–	±
α2	150	+ or –	–	–	±
ϕ	8	+	+	ND	ND
E22	ND	+	+	ND	ND
E60					
Lower 1 mL	ND	+++	++	+	+
Pellet	ND	+	+	+	+
Strain X73I (capsule)					
Formalin killed					
A1	ND	+	–	+	–
A2	ND	+++	+++	++	+++
A3	ND	++	++	++	++
ϕ	ND	++	++	++	++
No formalin					
α1 + α2 (combined)	ND	+	–	–	±
LPS extracted by PCP procedure					
Strain X73B (no capsule)					
Formalin killed	0				
No formalin – P	~2%[e]	+++	++	++	+++
Strain X73I (capsule)					
Formalin killed	0				
No formalin – P	~2%[e]	+++	++	++	+++

[a]Abbreviations used A1, antigens in aqueous layer after first partitioning of FK cells with phenol–water; A2, antigens in aqueous layer after re-extraction of the phenol layer and precipitate with water; ϕ, antigens remaining in phenol after completion of extraction; α1, antigens in aqueous layer after first partitioning of NF cells; α2, antigens in aqueous layer after re-extraction of phenol layer and residue with water; E-22, antigens extracted by EDTA at 22° from phenol–water precipitate obtained by phenol–water extraction of nonencapsulated NF cells; E-60, antigens extracted from foregoing residue with EDTA at 60°; α1 + α2, combined antigens in the two aqueous layers after phenol–water extraction of NF cells; P, PCP-LPS. [b]Milligrams dry weight of water-soluble fractions after dialysis per 40 g wet weight of cells. The weights of insoluble residues were not measured. [c]Details on the preparation of the antisera are given in Methods. [d]–, no discernible line at all; + or –, lines obtained with some extracts and some antisera, but no lines observed with others; ±, very weak line; +, ++, +++ progressively stronger lines; ND, not done. [e]Percentage LPS based on weight of alcohol–ether dried cells.

A2 gave strong lines in immunodiffusion, and the combined aqueous layers $\alpha 1$ + $\alpha 2$ (Table I) gave either a weak line or no line at all, depending on the antisera.

PCP extraction. — Alcohol–ether-dried preparations of *P. multocida* X73I and X73B were extracted by the PCP procedure[10]. No LPS was extracted from FK cells, but ~2% LPS was obtained from NF cells (Table I). Chicken antiserum to the PCP-LPS from *P. multocida* X73B was then prepared[13] and tested by immunodiffusion (Table I).

Immunodiffusion comparison of Westphal and PCP-LPS . — The Westphal LPS fractions and the PCP-LPS were evaluated by immunodiffusion with antisera prepared with LPS obtained from FK or NF *P. multocida* X-73B cells (Fig. 1). Strong lines of reaction were obtained with A2 and P, weaker lines with A1, and the weakest lines of all with $\alpha 1$ and $\alpha 2$. Reactions of fusion indicative of antigenic identity were obtained between A2 and P. Spur formation may be seen (suggesting partial antigenic identity) between $\alpha 1$ and $\alpha 2$ with P or A2, and also between A1 and A2 or P.

Almost identical results were obtained with similar LPS extracts from the encapsulated strain X73I as with those from the nonencapsulated X73B.

Ultracentrifugation of extracts. — When fractions $\alpha 1$, $\alpha 2$, A2, and P were ultracentrifuged under conditions usually used to pellet LPS, namely 2 h at 105,000*g*, the serological activity of the A2 and P fractions was concentrated in the pellet, but the serological activity of $\alpha 1$ and $\alpha 2$ remained in the supernatant solution.

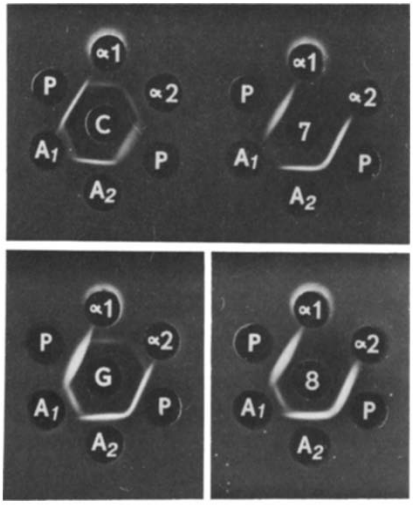


Fig. 1. Reaction of Westphal extracts and PCP-LPS with avian antisera. *Outer wells:* $\alpha 1$, $\alpha 2$, antigens from first and second aqueous Westphal extracts from *P. multocida* X73B NF cells; A1, A2, antigens from first and second aqueous Westphal extracts of FK cells; P, LPS extracted by the PCP procedure from *P. multocida* X73B NF cells. *Inner wells:* C, antiserum prepared in chicken with X73I FK cells; G, antiserum prepared in chicken with PCP-extracted LPS; 7, 8, antisera prepared in checkens with LPS extracted by EDTA from *P. multocida* X73B NF cells.

TABLE II

CHEMICAL COMPOSITION OF *Pasteurella multocida* FRACTIONS

Fraction[a]	*Glucose*	*Galactose*	L-glycero-D-manno-*Heptose*	*3-Deoxy-*D-manno-*octulosonic acid*	*Ribose*	*Tetra-decanoic acid*	*2-Hydroxy-tetra-decanoic acid*	*Fraction*	*GlcN*	*Lys*	*Gly*	*Ser*	*Asp*
X73B A1	2.73%	3.24%	1%	0.75%	13.3%	ND	ND	X73B A1[c]	2.84%	ND	0.56%	ND	ND
X73B A2	2.60	3.06	6.5	0.72	8.94	ND	ND	X73B A2[c]	3.72	ND	0.78	ND	ND
X73B ϕ	5.4	3.96	13.3	0.58	0.74	ND	ND	X73B ϕ[c]	3.29	ND	1.18	ND	ND
X73I A1	ND	ND	ND	ND	ND	0.03%	0%						
X73I A2	4.0	5.0	10.6%	ND	ND	0.90[b]	2.3[b]						
X73I A3	0.94	1	2.51	ND	0.74	3.9	5.9	X73I A3	5.29	1.13%	0.58	0.55%	0.70%
X73B α1	1.04	1.66	0	0.06	13.8	ND	ND	X73B α1	2.27	ND	0.55	ND	ND
X73B α2	1.57	2.60	0	0.34	8.3	ND	ND	X73B α2	2.48	ND	0.38	ND	ND
X73B ϕ	4.14	3.6	10.7	0.08	None	ND	ND	X73B ϕ	2.47	ND	0.78	ND	ND
X73B EDTA-60	1.45	0.74	2.42	ND	2.25	ND	ND						
X73I α1 + α2	2.4	2.9	0.6	0.4	6.9	Trace	Trace						
X73B P	4.52	4.49	14.1	1.84	None	7.6	14.0	X73B P[c]	6.36	2.66	2.45	0.99	0.34
X73I P	3.78	3.01	10.4	1.60	None	8.0	13.3	X73I P[c]	5.29	3.58	0.73	1.31	0.48

[a]See key in Table I. [b]Second preparation of A2–CH_2O, 2.7% tetradecanoic acid, 4.0% 2-hydroxytetradecanoic acid. [c]A peak corresponding to 2-aminoethanol was found in these fractions.

Chemical analyses of fractions. — The serologically most-active fractions, X73I-A2, X73B-A2, X73B-P, and X73I-P, contained about twice as much heptose as glucose or galactose; the least-active fractions A1, α1, and α2 contained little or no heptose (Table II). In the capsulated variant, the data show that tetradecanoic and 2-hydroxytetradecanoic acid are present in the serologically active fractions A2 and P, and absent from the serologically inactive fractions (α1 and α2). Ribose was found in A1, A2, α1, and α2, presumably as a result of contamination with RNA. All of the fractions were analyzed for 2-deoxy-*erythro*-pentose by the diphenylamine procedure, but only the EDTA-60 extract contained this sugar (suggestive of DNA). After digestion with DNAse and RNAse, the mixture was centrifuged at 105,000*g*. Although the upper 9 mL was serologically inert, the pellet and a fraction in the lower 1 mL of the centrifuge tube were serologically active.

TABLE III

TOXICITY OF X73B-LPS FRACTIONS TO 10-DAY-OLD CHICKEN EMBRYOS

Fraction	LD_{50} (μg)	*95% confidence limits*	
		Lower	*Upper*
Formalin killed			
A1[a]	8.1	3.5	19.2
A2	0.52	0.12	1.76
ϕ	0.025	0.0046	0.104
No formalin			
α1	25.3	6.38	163.3
α2	3.64	0.34	321.1
ϕ	0.97	0.31	3.18
E60, not centrifuged	0.60	0.14	2.16
E60, lower 1 mL	0.39	0.15	0.97
E-60, pellet	13.5	ND	ND
P	0.0019	0.00054	0.0054

[a]Same fraction designations as in Tables I and II.

TABLE IV

LIMULUS AMEBOCYTE LYSATE ACTIVITY OF FRACTIONS

Fraction	*Minimum concentration*[a] *(ng/mL)*
A1	0.1–1
A2	0.001–0.01
α1	1–10
α2	1–10
P	0.01–0.1

[a]The two concentrations given denote the results of two different determinations for detectable gel formation.

Analyses of these fractions by the cysteine–sulfuric acid reaction[7] indicated that they were chemically different; the pellet contained only hexose and the lower 1-mL fraction contained both hexose and heptose. The fraction in the lower 1 mL had the greater toxicity (Table III), and the greater serological activity in immunodiffusion.

Chicken-embryo toxicity tests. — The A2 fraction from the phenol–water extracts of the formalin-killed cells was more toxic than the $\alpha 1$ or $\alpha 2$ fractions (Table III). The P-LPS was the most toxic.

Limulus lysate test for endotoxin. — The A2 fraction was more active than the $\alpha 1$ and $\alpha 2$ fractions (Table IV). The PCP-LPS was approximately equal in activity to the A2-LPS.

Electron microscopic examination. — Fractions A2, P, and $\alpha 1 + \alpha 2$ were stained with uranyl acetate and examined in an electron microscope. Fractions P and A2 contained the typical ribbon-like structures previously reported[19] for LPS. However, no structures characteristic of LPS were seen in fraction $\alpha 1 + \alpha 2$. Differences were seen in the structures between the P-LPS and the A2-LPS. The P-LPS ribbon-like structures were larger in diameter, and groups of spherical structures were present at the branch points; spherical structures were absent from the A2-LPS. Neither preparation was morphologically homogenous.

DISCUSSION

The initial screening for the presence of LPS was performed by immunodiffusion. The results obtained by this reaction are directly dependent on the specificity of the antisera used. Antisera of broad specificity, prepared with formalin-killed *P. multocida*, were used initially so that LPS-related antigens, as well as purified LPS, would be detected. These tests were followed with highly specific antisera prepared with purified LPS, so that only LPS would be detected in the extracts. The failure of the Westphal procedure to extract LPS from NF *P. multocida* strain X-73 cells and its success in extracting LPS from FK cells was unexpected. With *P. multocida* strain P-1059, formalin killing has no effect on the extraction of LPS by the Westphal procedure. The PCP procedure readily extracted LPS from strain X-73 NF cells but not from FK cells.

Cross-linking of hydrophilic chains to *P. multocida* strain X-73 LPS, mediated by formaldehyde, would increase water solubility of LPS and presumably extractability of LPS into the aqueous phases by the Westphal procedure, and at the same time decrease extractability by the PCP procedure. This may occur by reaction of formaldehyde with the amino groups of LPS to form "methylolamines"[22], and these could undergo further cross-linking reactions with hydrophilic molecules such as RNA or protein. Formaldehyde is known to react[23] with LPS, because extracting LPS from *Salmonella* bacteria that have been killed with $^{14}CH_2O$ produces LPS that contains ^{14}C. Salmonella LPS contains amino groups in the form of 2-amino-ethanol and 4-amino-4-deoxyarabinose in the lipid

A portion of the LPS. *Pasteurella multocida* LPS also contains 2-aminoethanol. Support for the concept that nucleic acids may be cross linked to LPS is given by ultracentrifugation behavior. In the usual case when LPS is extracted from cells that have not been killed with formalin, centrifugation at 105,000*g* pellets LPS, and nucleic acids remain in the supernatant solution. However, LPS and nucleic acids from formalin-killed *P. multocida* pelleted together when centrifuged[8] at 105,000*g*.

The lipopolysaccharides of *P. multocida* have been previously reported[24] to be of the R type, which lacks polysaccharide side chains and is less hydrophilic than S type. Erler's studies on *P. multocida* LPS were performed by the Westphal extraction of formalin-killed *P. multocida*[25]. Because the LPS from *P. multocida* cells that have not been treated with formalin were readily extracted by the PCP procedure, as are R-type LPS[10], it would appear that both the virulent encapsulated strain X73I and the nonencapsulated X73B have R-type LPS.

Antisera were prepared with LPS from FK and NF cells to evaluate the possibility that formalin killing altered the antigenic specificity of the LPS. Reactions of antigenic identify (fusion) were found between LPS prepared from FK or NF cells when they were examined with antisera prepared with either type of LPS. We concluded that formalin killing of *P. multocida* did not cause any apparent changes in the antigenic specificity of its LPS. The antisera prepared with the different preparations of LPS varied in specificity (Fig. 1). Because the EDTA–LPS antiserum reacted well with fractions A2 and P, and poorly or not at all with fractions $\alpha 1$ and $\alpha 2$, we consider it the most specific *P. multocida* LPS antiserum of those tested. There is good evidence to support the concept that A1, $\alpha 1$, and $\alpha 2$ contain very little LPS for their toxicities are much lower than A2 or P. Furthermore, A1, $\alpha 1$, and $\alpha 2$ contain very little heptose or fatty acids, components that can be used as markers for *P. multocida* LPS.

One possible explanation for the observation that the PCP-LPS is almost 100 times more toxic than the A2-LPS (Table III) is that the formalin killing of *P. multocida* partially detoxifies its LPS. Previous attempts to detoxify isolated LPS with formaldehyde have failed[26]. However, formalin-treatment of intact organisms containing LPS has resulted in potent vaccines of decreased toxicity[27]. The reaction of isolated LPS with formalin is likely to be different from the reaction of formalin with LPS in bacterial cells. The differences in the LPS structures observed between A2-LPS and P-LPS with the electron microscope suggest that formalin killing has some effect on their morphology.

In conclusion, formalin killing of *P. multocida* has been found to affect the extractability of LPS by both the Westphal and PCP procedures, based on the evaluation of LPS in the extracts by immunodiffusion, toxicity, chemical composition, and electron-microscopic examination.

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