# COMPARATIVE TOXICITY OF PASTEURELLA MULTOCIDA ENDOTOXINS FOR CHICKEN EMBRYOS

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
JAMES T. GARY, JR.
1971

ABSTRACT

COMPARATIVE TOXICITY OF *PASTEURELLA MULTOCIDA*

ENDOTOXINS FOR CHICKEN EMBRYOS

By

James T. Gary Jr.

Eleven *Pasteurella multocida* strains selected to represent isolates from 5 different animal sources were extracted by 2 separate methods and their lipopolysaccharide antigens purified. Ultraviolet absorption at 280 and 260 mμ indicated that all extraction products were essentially free of protein and nucleic acid contamination. The poorly-immunogenic nature of these preparations was indicated by their failure to produce a detectable antibody response in rabbits. Lethal doses of the purified lipopolysaccharides for young mice were high with $MLD_{50}$ values greater than 200 μg., and the localized Shwartzman reaction could be evoked in rabbits by only about half of the lipopolysaccharides tested.

Intravenous inoculation of 11-day-old chicken embryos resulted in $CELD_{50}$ determinations which differed 125-fold for the 12 endotoxins examined. The endotoxin from a strain of *P. multocida* isolated from a dog was the least toxic of all preparations, and differed significantly from toxins extracted from strains representing the other animal species. No significant differences could be detected in the toxicity of lipopolysaccharide components of strains from 3 of the 5 animal sources when examined by the chi-square ($X^2$) contingency method of statistical

analysis. Significant differences were found, however, between extracts from the avian *Pasteurella*. The antigenic complexity of *P. multocida* is discussed.

# COMPARATIVE TOXICITY OF *PASTEURELLA MULTOCIDA* ENDOTOXINS FOR CHICKEN EMBRYOS

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department Microbiology and Public Health

1971

ACKNOWLEDGEMENTS

I would like to sincerely thank Dr. G. R. Carter for his guidance and immeasurable assistance in the development and conduct of this study. Grateful acknowledgement is also made to Mr. A. Wayne Roberts for his assistance with many technical operations, and to Mr. Roberts, Mrs. Ann Steketee and Mrs. Dorothy Boettger for their individual efforts in helping to secure necessary supplies and other materials.

Finally, I am deeply indebted to Dr. Charles W. Johnson for his expert construction of the figures used in this thesis and to Harold and Pamela McAllister for the excellent photographic reproductions which made this work complete.

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# INTRODUCTION

The etiologic agents of hemorrhagic septicemia, fowl cholera, swine pneumonia and other pasteurelloses are presently thought to be a closely related group of organisms, all of the same species, which has been named *Pasteurella multocida*. Early investigators rejected the idea of a single species and for some years a zoological classification based on the animal source of the organism was adopted. Although a close relationship between the bacterial strains, with respect to such factors as group constituents (5), cross-pathogenicity (79) and cross-immunity (73,77,79) was recognized by many later workers, the zoological classification remained the one most frequently used until about 1939. Rosenbusch and Merchant conducted a comprehensive study including an attempt to correlate all the available literature and suggested that all of the "typical," nonhemolytic, hemorrhagic septicemia *Pasteurella* be grouped into a single species to be called *P. multocida* (114). They further suggested that these organisms be divided into subgroups on the basis of agglutination reactions and fermentation of xylose, arabinose and dulcitol (114). For the most part these suggestions were adopted and the name *P. multocida* was widely accepted. Additional studies ensued and there were several attempts to demonstrate a strict host-specificity among the *Pasteurella* examined; although none was successful. Serological studies were conducted by several workers (18,19,65,66,113) and strains were typed on the basis of capsular antigens and passive

immunization as well as cross-protective ability and agglutination properties.

That hemagglutination and mouse protection tests were inadequate for complete typing of *P. multocida* was suggested by Bain and Knox (3) after strains belonging to the same serotype were found to differ in lipopolysaccharide composition and virulence for cattle. Studies on the somatic antigens of these organisms led to the elucidation of 11 apparently distinct serotypes (85,86), based on combinations of O and K antigens. A more profound indication of the importance of somatic antigens to a serological classification was evidenced when strains of the same capsular type but differing in their capacities to elicit hemorrhagic septicemia in cattle were shown to contain different O group antigens and, conversely, when strains of different capsular types were reported to possess common O group antigens and similar pathogenicity for cattle (86). Two interesting correlations became evident during the studies of Namioka and Murata. Only strains possessing the O group antigen given the numerical designation "6" were shown to produce hemorrhagic septicemia in cattle naturally; with the capsular type of the organism being of no apparent consequence in its induction. Among those strains responsible for fowl cholera in chickens and turkeys, however, at least 2 distinct O-antigenic types are common while only a single capsular type (Carter's type A) has been found. No significant relationship between antigenic makeup and host affinity has been noted among *Pasteurella* isolated from other animals since several different serotypes have been found to cause pasteurellosis in most of them. Prince and Smith studied several strains of *P. multocida* and reported that distinctions between somatic antigens corresponded with those predicted from known O somatic types (107), thereby confirming the work of Namioka and Murata. Hence, the classification based on somatic

and capsular antigens of *Pasteurella multocida* has given additional strength to the case against a specific host-species for which the different strains within this group are pathogenic.

Smith and Thomas (126) found intravenous inoculation of 10- or 11-day-old chick embryos with very small quantities of lipopolysaccharide from several different bacterial species to be an extremely sensitive assay method for endotoxin. Finkelstein subsequently verified the sensitivity of this assay method and also its reproducibility (27). Milner and Finkelstein (78), in an extensive study, correlated chick embryo lethality with pyrogenicity in rabbits. These authors reported that while a direct correlation exists between the 2 assays, the chick embryo method is faster, less tedious and much less expensive to perform.

The acute sensitivity of the chick embryo lethality test has been used in this study to detect any differences which might exist in the toxicities of purified lipopolysaccharide antigens from the cell walls of several strains of *P. multocida*. While many of the former investigations have been conducted with crude extracts containing nucleic acid and denatured protein, as well as capsular polysaccharide contaminants, it appears immediately obvious that comparative studies with preparations containing variable amounts of such substances are necessarily inaccurate and of little validity. Therefore, in the following experiments vigorous extraction procedures, designed to eliminate protein and nucleic acid contaminants, have been used. Washing the cells with saline prior to extraction, selection of noncapsulated variants, where possible, and fractionation of the extract with ethanol have been utilized in an attempt to remove all capsular and other extraneous polysaccharide material, especially in those extracts not subjected to ultracentrifugation.

Since the earlier observations mentioned above indicate that capsular components of *P. multocida* cannot be implicated directly in the various animal diseases caused by this agent, it was thought that their role may be almost entirely a protective one. If this were true, then the presence of capsular substances among components for which true toxicities were being determined would be both unnecessary and undesirable. If, on the other hand, cell wall components play a major role in the pathogenesis of *Pasteurella* infection, pure preparations extracted in the same manner and administered in equivalent amounts would be expected to have statistically insignificant differences in toxicity. Ribi *et al.* (32,111) have stated that no correlation could be found between biological properties and gross chemical composition in purified extracts from *S. marcescans* and *E. coli*. It seems relevant to mention, however, that mice were used by these researchers for determination of toxicity and it is conceivable that in the dose ranges required for such determinations small, but significant, differences may have been masked by the large differences between the amounts of material in successive dilutions.

The chick embryo system, by virtue of its response to fractions of a microgram of lipopolysaccharide would appear to be ideally suited for demonstrating either minute or vast differences in toxicity. It was in this spirit that the experiments reported below were performed. Since the toxic doses were very limited in range for all the preparations tested, statistical evaluation by the $X^2$ contingency method has been applied to the data obtained, in order to detect significant differences in toxicity among the various strains. The relationship of any existing differences to animal host is discussed.

# REVIEW OF THE LITERATURE

## Studies on the Chemical Nature and Physical Properties of Endotoxins

### General Characteristics of Somatic Antigens

The biologically active "somatic antigen" found in the cell walls of most gram negative bacteria is commonly referred to as endotoxin. This nomenclature is principally due to its constituitive role within the cell walls of such organisms and to its toxicity upon administration to animals. Much disagreement still exists as to the exact chemical components which comprise the intact endotoxin molecule. Boivin (14) was the first investigator to extract the molecule from unaltered bacterial cells and the complete antigen was found to be a lipopolysaccharide-protein complex. This is presently referred to by some authors as "Boivin-type" antigen. Many notable contemporary investigators regard the endotoxic moiety as lipopolysaccharide (2). This component of the somatic antigen is described by Westphal *et al.* (138) as having a molecular weight of approximately $10^6$ and endotoxic as well as O-antigenic properties.

The numerous studies of Westphal, Lüderitz and their co-workers and of many other investigators have clearly demonstrated that lipopolysaccharides are long-chain, phosphorous-containing heteropolymers composed of a lipid (lipid A) covalently linked to a core polysaccharide to which are attached O-specific, serologically different oligosaccharide units. The polysaccharide components of lipopolysaccharide complexes have been

found to vary greatly in composition over the range of organisms in which they have been studied. Such variations within the serologically active O-specific side chains of the somatic antigens from *Salmonella* form the basis of the widely acknowledged Kauffmann-White classification scheme (55,56).

Protein Component of the Complete Somatic Antigen

Dependent upon the method of extraction, 2 different forms of the protein component of complete somatic antigens are obtainable. Morgan and Partridge (80,81) observed that when the whole antigen complex is split off from the cell wall with 1% acetic acid at 100 C., the product contains lipid B, degraded polysaccharide, and "conjugated protein." This "conjugated protein" upon treatment with 90% phenol followed by ethanol precipitation yields a "simple amphoteric protein," similar to that obtained when the complete antigen complex is dissociated with phenol or alkali (80,81). Lipid A is found in association with the conjugated protein and it is widely believed that the 2 proteins are distinct, although no experimental proof has been cited (70). The simple protein has no toxic properties although it has been demonstrated by Goebel (15) that, in addition to other biological properties, the conjugated protein is highly toxic for mice. In being devoid of biological activity, the simple protein resembles degraded polysaccharide. This relationship may well be due to the absence of lipid A from both molecules (70).

Taylor (129), Pershin (99) and Webster (135) have all found that although lipopolysaccharides exhibit O-specificity and are potent endotoxins, they are weak antigens due to the absence of the protein component. In experiments with *Shigella dysenteriae*, the isolated "conjugated

protein" was shown to be immunogenic when injected into rabbits (81). The lipopolysaccharide of this organism was almost totally devoid of immunogenicity when compared with the whole antigen complex. However, when the conjugated protein and lipopolysaccharide were recombined, an artificial complex resulted which induced the formation of O-specific precipitins and agglutinins (81). More recent studies (123,134,31) have indicated a role of greater functional importance for the protein component of complete somatic antigens in (a) the establishment of delayed-type hypersensitivity and (b) increased host reactivity to subsequent challenge with endotoxin, than had been previously attributed to them by many researchers (1,145) (see below).

Lipids Associated with the Complete Somatic Antigen

It has been determined, on the basis of their chemical nature and biological activity, that 3 distinct lipid fractions related to the somatic antigen are present in the cell envelopes of gram negative bacteria (53,67). These are lipid A, lipid B, and a third lipid, distinguished much more recently, the role of which remains largely undetermined (53, 67). Lipid B is a loosely linked component of the complete antigen complex which can be found in the product after dissociation with dilute acetic acid (15,80,81). It can also be obtained in a yield of about 10% by dissociation with formamide or alkaline-ethanol. Not known, however, is whether lipid B specifically differs, with respect to chemical composition or biological activity, from other cephalin-like lipids found in cell wall or membrane (15,81). A role for lipid B in the biosynthesis of O-antigens has been indicated.

Lipid A was so named by Westphal *et al.* (137) to distinguish it from the more easily removed lipid B. It is covalently linked within

the lipopolysaccharide, from which it (along with degraded polysaccharide) may be obtained by mild acid hydrolysis (88,138). The results of studies on a variety of bacterial groups indicate that lipid A preparations are very similar with respect to general structure and composition (70). In spite of the vast amount of research on the structural analysis of lipid A, it is still generally agreed that the exact structure remains undetermined. Many of its constituents have been determined, however, and it was demonstrated by Nowotny that 20 to 22% of this material is comprised of D-glucosamine, probably in a phosphorylated form (88). Ikawa *et al.* (50,51) identified lauric acid, myristic acid, palmitic acid and D-β-hydroxymyristic acid as fatty acid constituents of lipid A from *Escherichia coli*. Qualitative analysis by paper chromatography showed nearly identical patterns of spots for fatty acids hydrolyzed from the lipopolysaccharides of 11 *Salmonella* and *E. coli* strains.

On the basis of their experimental data, Nowotny (88) and Burton and Carter (16) have concluded that lipid A is constituted by a backbone composed of n-β-hydroxymyristoyl-glucosamine-phosphate, in which all the available -OH groups of the glucosamine residues, as well as those of the hydroxy acids, are esterified by long-chain fatty acids and possibly acetic acid. The position of the phosphate group has not been determined and nothing is known about the nature of the linkages residing between the glucosamine residues. Existing data seem to indicate an equal likelihood of either a glycosidic bond or a phosphodiester bond (16,88).

From studies on the lipopolysaccharide of *E. coli* 0111, Heath and Ghalambor (37) identified 2-keto-3-deoxyoctonic acid (KDO) as one of its components. Osborn (93) found that this compound was also present in the "core" polysaccharide of a mutant strain of *S. typhimurium*. After observing that about 25% of the KDO present can be found at the reducing

terminal end of glucose-heptose-phosphate chains, Osborn suggested that it may be involved in the linkage of the core polysaccharide to the lipid A moiety of the intact lipopolysaccharide (93). Edstrom and Heath (25) obtained a fragment of lipid A which functioned as an acceptor for the incorporation of KDO from CMP-KDO and showed the linkage to be ketosidic. This observation lends further support to the suggestion that KDO may function as a linkage group.

The biological role of lipid A in endotoxic reactions has been frequently investigated, yet it remains only partially elucidated. Goebel (15) was the first to postulate the existence of a toxic component of endotoxins which will endow either protein or polysaccharide with toxicity, depending on the method used for isolation. It is now well established that lipid A is responsible for toxicity, but no one knows to what extent. The discovery that lipid A-poor endotoxins--as isolated by Ribi *et al.* (111)--are highly potent has led to the generally accepted belief that no quantitative relationship exists between toxicity and lipid content. On the other hand, at least 2 important observations have left little ground for doubting that a qualitative relationship exists. In the first instance, preparations completely devoid of lipid A (e.g., degraded polysaccharides and simple proteins) were found to be nontoxic (111). Of greater consequence, however, was the isolation by Lüderitz *et al.* (68) of mutant strains of *Salmonella* from which they obtained endotoxic "lipopolysaccharide" containing mainly lipid A and no polysaccharide.

Polysaccharides Found in Somatic Antigens

It has been indicated previously that both protein and lipid components of the somatic antigens from a variety of gram negative bacteria

have been found to be chemically similar. No such similarity exists among the polysaccharides of these organisms. Sugar composition and structure vary within species as well as genera and families. That the polysaccharides of enterobacterial somatic antigens are the carriers of their serological O-specificity has long been recognized and the variation among these components parallels the great variety of serological specificities which have been observed (59,60). Many studies have dealt comparatively with composition and specificity by analysis of sugars (70,69,68,92), especially those of *Salmonella*. A major observation has been the correlation between classification of *Salmonella* serotypes into serogroups and the sugar composition of their respective O-antigens (57,69). Similar correlations were also determined for other organisms of the Enterobacteriaceae (70). Continued analysis and evaluation of the polysaccharide structure of these organisms has now led to the association of serological specificity with small discrete polysaccharide units known as determinant groups (70).

Current concepts on the role of KDO in lipopolysaccharides have already been mentioned. In all studies reported to date the reducing-terminal carbohydrate found linked to KDO has been a phosphorylated heptose. Along with n-acetyl glucosamine, galactose, and glucose, heptose phosphate and KDO are thought to form the "core polysaccharide" of *Salmonella* (69). Among the presumptive evidence for this assumption is the fact that these 5 basal sugars are components of the O-antigens of all *Salmonella* and, further, they constitute the R II polysaccharides (lipopolysaccharides of mutant *Salmonella* lacking O-specific side chains) of Kauffmann *et al.* (57,58,69). Hence, the basis has been provided for some to tentatively hypothesize a constant polysaccharide composition within the basal core region of each genus of gram negative bacterium.

More than 20 constituent sugars have now been found in O-antigens from a variety of bacteria (70). Specific groups which have been identified are hexoses, 6-deoxyhexoses, 3,6-dideoxyhexoses, heptoses, hexosamines, pentoses, and 2-keto-3-deoxyoctonic acid (KDO), although not all of these are frequently encountered. As it has been indicated (above) the most common are heptose, KDO, galactose and glucose, while pentoses are very rarely found. The O-specific polysaccharides of gram negative bacteria are heteropolysaccharides which generally contain 5 or more sugar constituents and, though more than 40 different sugar combinations (chemotypes) have been found in specific polysaccharides, several combinations have never been encountered together. Among these are included two 3,6-dideoxyhexoses and fucose together with a dideoxyhexose (70,69).

Since most early characterizations utilized paper chromatography for detection and identification of sugars, it is thought that in many cases the presence or absence of a spot may have formerly been misinterpreted. In addition, just as KDO and heptose were not tested for in earlier investigations, other sugars might yet remain obscured. Hence, it is likely that with the present methods of analysis and sequencing by means of gas liquid chromatography, other previously undetected sugars may be identified in the future.

On the basis of work done by several groups of researchers (38,47, 68,69,70,92,94) it has become possible to formulate models for the structure of complete lipopolysaccharides as well as for constituents of the various regions. One such model is diagrammed in Figure 1 (139).

It was indicated earlier that the exact position on the glucosamine unit of lipid A to which the KDO trisaccharide of the inner core is bound ketosidically is still not clearly established. Westphal *et al.* (139) also posed several other questions which remain to be elucidated. Among

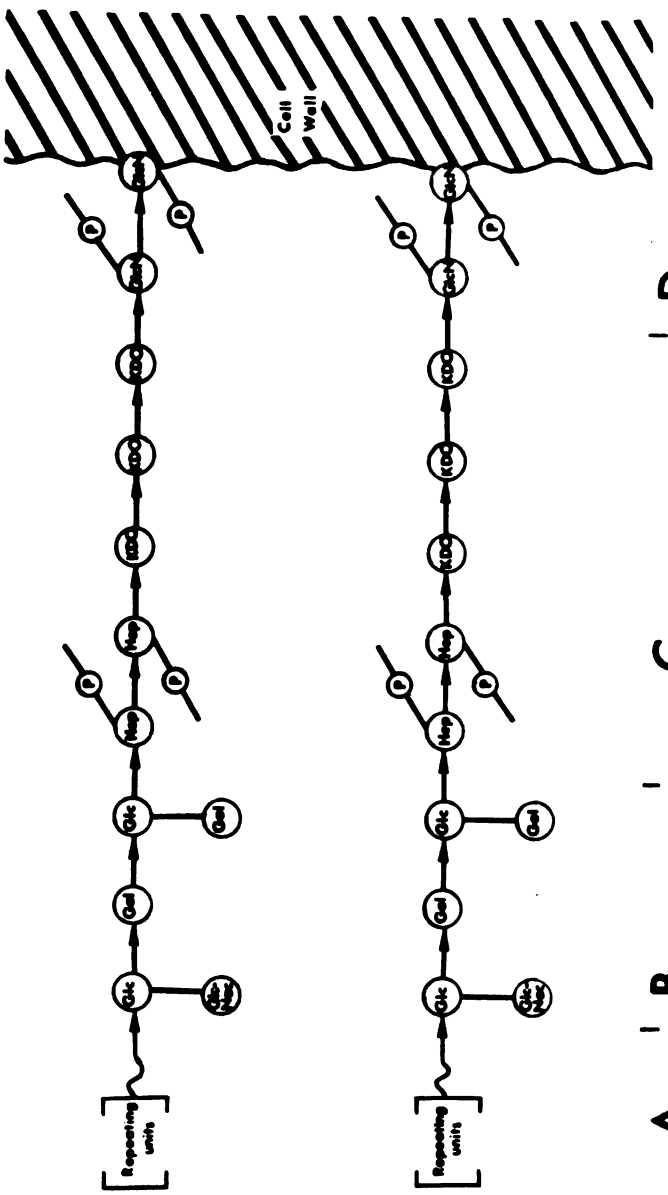



Fig. 1. Simplified structural model of *Salmonella* lipopolysaccharides. From: Westphal, *et al.* (139).

these are: (a) what kind of linkage exists between lipid A and cell wall protein which is split by hot phenol-water? and (b) how ethanolamine phosphate is bound within the glycolipid? From investigations of the mode of attachment between protein and lipopolysaccharides and cell wall mucopeptides, it has been shown that isolated cell walls contain protein which may be removed by detergent extraction or protease digestion (112). Some of the proteins present have been shown to be enzymatic in nature (72,87, 128). In contrast to most gram positive bacteria, however, no mucopeptide components have been reported to occur in polysaccharides isolated from gram negative bacteria, nor does evidence exist to indicate that covalent bonds bind somatic or capsular polysaccharides to the mucopeptides or proteins of gram negative cell walls (140,141). The most widely held concept suggests that lipopolysaccharides and capsular (K) antigens are linked to the cell wall lipid-mucopeptide-protein primarily by physical bonds, either hydrophobic, physical, or both (141).

Dissociation of the Lipopolysaccharide Molecule

Oroszlan and Mora (91) were the first to show that treatment of *Serratia marcescens* endotoxin with the detergent sodium lauryl sulfate (SLS) resulted in dissociation of the particles into subunits no longer having the tumor-damaging potency of the parent molecules. According to them when the SLS was removed reaggregation of the subunits appeared to occur and toxic particles were again formed. In 1964 Skarnes and Chedid (125), using chromate-51-labeled endotoxin, observed that it is degraded and inactivated both *in vivo* and *in vitro* by components of normal serum. Based on these studies a 2-step mechanism leading to the inactivation of endotoxin in whole serum was proposed. It was thought to involve an initial step of degradation followed by a second step of detoxification;

the latter being limited by the rate of degradation which, in turn, is dependent on the calcium level of serum. Rudbach conducted similar studies on the effect of binding by plasma proteins on endotoxin activity (118). He, in conjunction with Ribi and a group of co-workers (112), also examined the conditions under which a second surfactant, the bile salt sodium deoxycholate (NaD), would depolymerize endotoxins from several species of bacteria. These experiments led to a physical characterization of the subunits produced, as well as a correlation of their occurrence with a reduction in pyrogenicity of the preparations and the discovery that minute quantities of human plasma would stabilize the subunits and permit dilution of the NaD without reaggregating the subunits into pyrogenic endotoxin (112). A new model was then proposed for the structure of endotoxin and for its disaggregation and the subsequent binding of its elements by plasma proteins. In order to facilitate a clearer visualization of the relationship of this model (to the discussion to follow) a diagram of the proposed model is shown (Figure 2). Since treatment of the endotoxin with bile salts was carried out under conditions not thought sufficient to cause breaking of any covalent or primary chemical bonds, it is assumed that secondary or tertiary forces must be responsible for binding the elements (nonpyrogenic basic subunits) together in the toxic unit (112).

In a subsequent study of the reversible dissociation of endotoxin, Rudbach *et al.* (119) discovered that when a mixture of 2 endotoxins is dissociated and then allowed to reassociate, the subunit chains recombine heterologously and hybrid endotoxin molecules are formed that contain subunits from both of the endotoxins in the original mixture. These findings led to an attempt to hybridize endotoxin lipopolysaccharides with nontoxic, acid hydrolyzed haptenic polysaccharides and with native

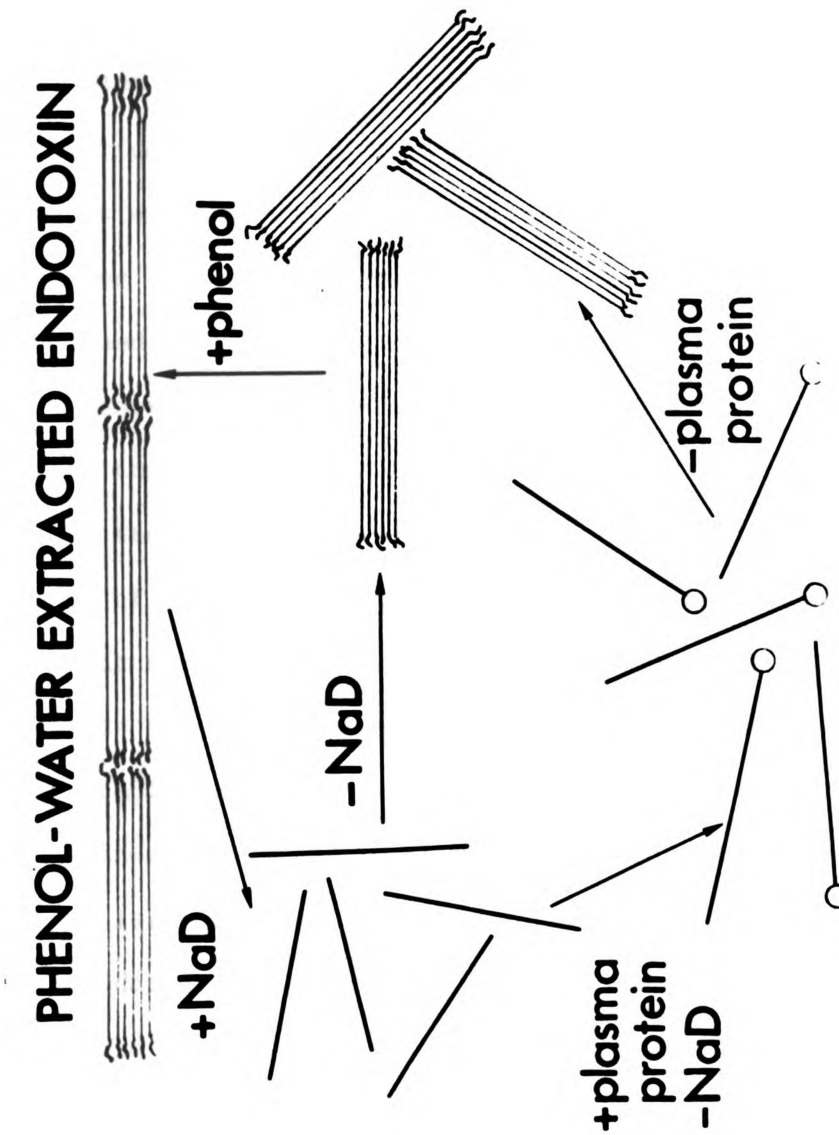



Fig. 2. Schematic diagram of the working hypothesis to explain the reaction of endotoxin with NaD and plasma (112).

protoplasmic polysaccharide. Lack of success in these attempts led to the proposal that only endotoxins could be induced to hybridize with endotoxins in this system and, further, that all endotoxins should be able to hybridize with one another (120).

To test this hypothesis endotoxins from smooth strains of *E. coli* and *S. enteritidis* and from the Re (heptoseless) mutant strains of *S. typhimurium* and *S. minnesota* were used. The results showed that formation of hybrids could be induced between the toxic extracts from heptoseless mutants and those from smooth strains of both organisms (120). Several other important observations were also made during this study. These included: (a) the occurrence of some degree of spontaneous hybridization in control mixtures of the Re endotoxins and endotoxins from smooth bacteria in the absence of NaD; suggesting that endotoxin in solution exists in a state of equilibrium with its dissociated subunits, but in the absence of surfactants the associated form is predominant; (b) demonstration that the minimal concentration of NaD needed to induce hybrid formation was the same minimal concentration that would dissociate the endotoxin complex and result in detoxification (120). This finding supported the theory that the toxic complex is formed by the specific association of at least 2 subunits; and (c) the formation of a hybrid among 3 endotoxins, confirming data which indicated that the endotoxic complex contained more than 2 subunits (119). Further investigations on the formation of hybrids among endotoxins are now in progress.

## Biological Action of Endotoxins

### *In vivo* Effects

The biological effects of endotoxins have been studied in a variety of animals, including horses and cows, and the literature is now saturated

with papers describing these varied effects. Some of the effects of endotoxin on animals noted by Osborne (95) were chill, biphasic fever, respiratory distress, depression, hypotension, cold extremities, anorexia, oliguria and anuria. Zahi and Hunter (144) and Berry (12) noted the occurrence of hypothermia in mice and Berczi (10) reported vomition in cats as a characteristic effect. In rabbits, leukopenia was found after administration of endotoxin by Bennett and Beeson (8), while Thomas and Jones (130) noted that leukemia in rats was cured after administration of this substance. In a qualitative study, Berenheimer and Schwartz (11) found a correlation between the hemolytic property of a toxin and its capacity to disrupt lysosomes, after it had been observed by Cohn and Morse (24) that endotoxin stimulated the phagocytic activity of rabbit leukocytes. Berry (12) noted upon administration of endotoxin from *S. typhimurium* to mice, that in addition to the drop in temperature, complete depletion of carbohydrate resulted. A rise in plasma histamine after toxin injection was reported by Hinshaw (44) to occur in dogs. Kuida *et al.* (62) examined the reaction of several species to endotoxin and little variation was found to occur between species such as the cat, rabbit and monkey.

Nykiel and Glaviano (89) observed that endotoxin stimulates the adrenal medulla in the dog with a resultant increase in epinephrine in the adrenal venous blood and Palmerio *et al.* (96) later found myocardial injury in addition to an increase in cathecolamines in the blood of rabbits following endotoxin administration. Circulating endotoxin was found by Herring *et al.* (43) to be distributed between the plasma and blood platelets in rabbits while a splanchnic pooling of blood was noted by Hinshaw (45). The dog has been the most frequently and intensively studied domestic animal and several other effects have been noted in

them. These included the observation by Petersdorf and Schulman (100) that injected endotoxin is taken up by the reticuloendothelial system (RES) and the discovery by Muller and Smith (82) that during shock in the dog, a rise in hematocrit occurs, along with an immediate fall in blood pressure, cardiac output and central venous pressure.

After studying the effect of prior experience with living or dead bacteria on the response of mice to endotoxin, Schaedler and Dubos (123) reported that mice free of ordinary mouse pathogens as well as intestinal *E. coli* and *Proteus* bacilli were highly resistant to the lethal properties of endotoxin. Following investigations on the fate of this substance, Lemperle (63) disclosed that endotoxin injected into mice is taken up almost exclusively by the RES and death occurs after failure of this system to remove circulating toxin. McKay *et al.* (74) examined the effects of endotoxin on the blood vascular system of rats and concluded that damage (*in vivo*) is induced by a triggering of the clotting mechanism. In a comparison of effect in relation to strain of organism, Carroll *et al.* (17) concluded that the immunologic, physiologic and pathologic responses of animals to administration of endotoxin are the same regardless of the species of gram negative organism from which the material is obtained.

A limited number of investigations have been conducted with large animals. Schalm found that several responses were evident in many large animals after the injection of endotoxin. These were characterized first by arteriolar constriction, fever, hypoglycemia and leukopenia followed by leukosis. In the horse, collapse resulted within 1 hour after initial intraperitoneal injection of the toxin (124). Halmagyi *et al.* (36) reported that in sheep, respiratory arrest is an important aspect of endotoxic shock, while Lillehei and Maclean (64) noted plasma loss, increase

in hematocrit, increase in plasma hemoglobin and necrosis of the bowel during shock in man. Endotoxin from *Pasteurella multocida* was reported by Heddleston *et al.* (41) to have brought about septicemia and death of calves within 24 to 48 hours. The gross and microscopic lesions resulting from acute infections and from administration of *P. multocida* have been studied by Rhoades *et al.* (109), who disclosed that endotoxemia caused by this organism is characterized by widely distributed hemorrhages, edema, general hyperemia and pneumonia.

In addition to the biological effects of endotoxin administration which have been summarized above, numerous others have been described in the literature. The most significant of these is based on the suggestive evidence of Kováts and Vegh (61), who concluded that endotoxins induce a specific hypersensitivity in certain animal hosts. This conclusion was prompted when they successfully elicited the Shwartzman phenomenon with heterologous endotoxin in rabbits made resistant to the homologous antigen. These researchers also noted the occurrence of seasonal variations in the sensitivity of mice to endotoxins (132), a phenomenon which had previously been observed by Berry (13). Several workers have described various effects of endotoxins on leukocytes normally involved in the cellular-immune system (42,97,143). These include cytotoxicity for mouse peritoneal leukocytes, inhibition of macrophage migration from mouse spleen explants, and stimulation of mouse spleen cell transformation.

A number of the more common biological effects of bacterial endotoxins are now routinely utilized in bioassays of these materials. Frequently employed is the pyrogenic (fever) response of rabbits to endotoxin administration. In addition, many investigators have made use of such effects as toxicity for various laboratory animals, protection of mice against bacterial infection, and elicitation of the local and/or

generalized Shwartzman phenomenon in bioassays of endotoxins from a host of different bacterial species. A pronounced lethality of gram negative lipopolysaccharides for intravenously injected 10- to 11-day-old chick embryos was first noted by Smith and Thomas (126) in 1956, and since has gained increasingly in popularity among researchers. Milner and Finkelstein have confirmed both the validity and the expediency of chick embryo lethality tests as a method for endotoxin assay (78), and it appears likely that this technique will be utilized on a routine basis by future investigators.

Mechanisms of Endotoxin Action

When one considers the diverse interests of researchers in the biological sciences it seems reasonable to assume that for virtually every substance known to be biologically active, there has been some attempt to elucidate its mechanism of action. Such is the case with endotoxin. The types of responses are so numerous, however, that many investigations have centered on the mechanism of production of individual responses as well as a general mechanism of endotoxic action. So closely do many of the biological responses to endotoxin resemble noninfectious disorders caused by other active agents, dietary injury and various hypersensitivity states, that a number of investigators have concluded that the mechanism of action, although it is not understood, is likely to be the same for endotoxin as for the agent inducing a similar response (9). Because of the wide range of effects and the inherent difficulties involved in investigations utilizing *in vivo* systems, few, if any, of the mechanisms of endotoxic action have been completely and unquestionably determined.

The experiments by Schaedler and Dubos (mentioned in the previous section), in which several strains of mice were used to examine the

contribution of enteric flora to the toxic consequences of endotoxin injection, resulted in the conclusion that at least 2 unrelated mechanisms are involved. Injection of small doses of toxin, according to the authors, elicits a primary toxicity which is characterized in mice by loss of weight and enhancement of infection, while an immunological reaction having lethal consequences is evoked in mice sensitized to endotoxin by prior exposure to gram negative bacilli (123). The concept that prior exposure to gram negative organisms is an important factor in susceptibility to endotoxin was reinforced in experiments by Freedman *et al.* (31), who demonstrated that modification of host reactivity, in the form of increased reactivity to subsequent challenge, required a protein component present in the preparations studied. Aqueous ether and Boivin-type extracts induced an incarese in (a) inhibition of water uptake, (b) the number of hemolysin-forming spleen cells and (c) skin reactivity when endotoxin was administered 10 days after pretreatment with these materials. None of these responses was increased when protein-free or Westphal-type preparations were used for pretreatment (31).

The 2-step mechanism proposed by Schaedler and Dubos was elaborated and further extended by Watson and Kim. These investigators proposed that the secondary toxicity is dependent on the acquisition of delayed hypersensitivity to a configuration which represents a related antigenic determinant present in the cell walls of most microorganisms, while the primary toxicity acts either independently or interdependently, depending on the immunological state of the host (134). The delayed hypersensitivity, during secondary toxicity, was said to be acquired as a result of clinical or subclinical infections and contact with the intestinal flora. Freedman *et al.* (31) indicated that if normal adult host reactivity to somatic antigens has an origin in acquired delayed hypersensitivity,

the presence of a protein or peptide determinant in the endotoxin macromolecule should be expected, as Watson and Kim had suggested. It was disclosed that the protein associated closely with endotoxic activity in the native state is present during natural exposure of the host to relevant bacteria (31). This protein was therefore assumed to represent the proposed "related antigen" (134) and the experimental results were said to support acquisition of delayed hypersensitivity as a step in the response to somatic antigens of gram negative cell walls. As somewhat of a supporting corollary, Stetson revealed that all the major effects of endotoxin, e.g., fever, shock and death, skin and corneal reactions and both types of Shwartzman phenomena, can be reproduced by antigen-antibody interactions in defined systems (127). It was his conclusion that the overall response to endotoxin has an immunological basis due to reaction of the toxic substance with "natural antibodies" within the host (127). This would suggest that the nature of endotoxin macromolecules is antigenic rather than intrinsically toxic.

The role of delayed-type hypersensitivity remains speculative, for although circulating antibody has often been postulated to, it has never been definitely shown to mediate the delayed sensitivity reaction. Fine (26) conducted a group of experiments on dogs and rabbits which led him to report that endotoxin causes death by its action on sympathetic nerves supplying the splanchnic viscera, especially those in the liver and intestine. Other properties of endotoxin were said to be uninvolved in the lethal outcome of a shock-producing dose. The injury to the splanchnic viscera is reportedly due to norepinephrine, or, more specifically, it is caused by norepinephrine produced locally in the injured tissues (26). A similar study conducted by Kass *et al.* (54) reported that there is an area in the posterior hypothalamus of the brain which possesses a

susceptibility to endotoxin of far greater degree than any other area of the body. Although endotoxin may not act directly on this site, any intermediary substances which might be liberated as a consequence of endotoxic action must, in their opinion, be liberated more readily in the brain than elsewhere. Cluff, in a recent review, indicated that the principal mechanism involved in transient decrease in resistance to infection following endotoxin administration is related to interference with granulocytic diapedesis and exudation, particularly in foci of bacterial localization. When bacteremia results, the decrease in resistance is attributed to interference with phagocytosis by the reticuloendothelial system (23).

## Host Response to Endotoxin: Resistance to Bacterial Infection

### Humoral and Cellular Responses

It has long been known that somatic antigens from gram negative bacteria, when administered to mice, can alter their resistance to infections by a variety of bacterial and viral agents. Small doses of lipopolysaccharide induce a rapid increase in the host's natural resistance to experimental infection. The increase is of short duration. Larger amounts of this substance, however, first elicit a phase of increased susceptibility which is followed later by increased resistance of somewhat longer duration (52). Rowley (116) was the first to suggest that the provocation of nonspecific resistance by bacterial lipopolysaccharides may involve at least 2 events. These were reported as (a) an increase in the opsonic capacity of the serum and (b) an increase in the inherent capacity of phagocytic cells to perform this function (116). It was observed by Rowley that in the normal animal the supply of opsonic constituents in the peritoneum is the limiting factor which

determines the outcome of the infection. The concentration of these opsonins needed to ensure phagocytosis varies with the virulence of the organism. Jenkin and Palmer acknowledged these principles after noting a rapid increase in titer of opsonins following intraperitoneal injection of lipopolysaccharide into mice. In this case, also, time of appearance and opsonin titer were dependent upon the amount of lipopolysaccharide injected. After all increases, however, there was enhanced removal and killing of bacteria within the peritoneal cavity and also faster clearance of bacteria from the circulation by the reticuloendothelial system (52). Whitby and his co-workers (142) were among other researchers of the period who acknowledged that both cellular and humoral changes occur within the host as a result of endotoxin administration. Howard and Wardlaw had reported (49) that the serum factors contributing to opsonic activity in the perfused rat liver are (a) specific antibody, (b) complement and (c) another heat-labile factor which might be properdin. A simultaneous report, however, indicated that although support for RES participation in nonspecific immunity was found, no correlation could be made between the stimulation of nonspecific immunity by lipopolysaccharides and the serum properdin level at the time of challenge (48). Whitby's group designed experiments to determine whether both humoral and cellular changes were of a nature that might reasonably account for the increased resistance which follows administration of endotoxin. No evidence was found for either cross-reacting antibodies or nonspecific cofactor-type substances and it was concluded that the increase in bactericidal titer after lipopolysaccharide injection in mice was due to a low level, general increase in specific antibodies to gram negative bacteria (75,142).

Rowley, in a very recent review, has suggested that the virulence of gram negative organisms is primarily dependent upon their resistance or susceptibility to phagocytosis and killing by the cells of the host. Gram negative cell walls are said to contain specific constituents which can activate the immune response into either humoral or cellular effector pathways. Differences known to exist between living and killed vaccines can therefore be attributed to the class of antibody induced on the one hand and to quantitative factors such as content of antigenic material and its rate of liberation on the other (117). With respect to a possible explanation for increased host resistance to unrelated organisms, the following points have been enunciated: (a) bacteria of all degrees of virulence exist even within the same serotype; (b) the external surfaces of virulent strains of *Salmonella* are probably dominated by the oligosaccharide antigens of the smooth-type lipopolysaccharide but have small areas of exposed surface protein and rough-core antigens common to many organisms; (c) specific immunization with killed vaccines or extracts produces antibodies against all of these surface antigens, which promote rapid phagocytosis; (d) provided the immunized animal has sufficient "active" phagocytic cells, these are probably capable of removing some of the cell wall lipopolysaccharides, "K" antigens, etc., and allowing the surviving antibody and complement to have access to the vulnerable bacterial membrane; (e) this process is greatly facilitated by the simultaneous action of intracellular enzymes such as lysozyme and phosphatases, and the bacteria eventually succumb to the combined enzymic onslaught; (f) avirulent organisms possessing the same lipopolysaccharide determinants must expose sufficient areas of the other common surface antigens to enable contact with host antibodies to occur (117). Thus, it appears conceivable that antibodies capable of opsonizing or promoting the

phagocytosis of many different species of gram negative organisms may result from contact with a variety of these organisms during the life of an animal, or from exposure of antigenic components of a single species which may be common to several different species. Endotoxin is known to behave as an adjuvant *in vivo*. Its action, then, may be a stimulation of rapid antibody production against previously contacted bacterial antigens, which, in turn, may enhance what has previously been termed "non-specific" resistance to infection.

Role of Complement in the Host Response

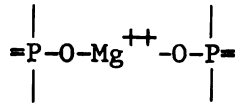
One notable point, following from the above discussion, is that even with the many investigations on bacterial endotoxins, very little conclusive evidence has been obtained on the nature of the biological mechanisms which govern the action of these ubiquitous substances. Equally little is certain about the mechanisms which govern the various host responses. Numerous experiments have been devoted to elucidating the role of complement in the host response to endotoxin. For the sake of brevity no detailed account of the majority of these investigations will be given. There are 2 relatively significant reviews on this area which appear to deserve comment, however. Both Rowley (115) and Wardlaw (133), as well as Michael and Landy (76), investigated the endotoxin content of serum-resistant and serum-sensitive strains of bacteria and noted that smooth, serum-resistant strains contain more lipopolysaccharide than rough, serum-sensitive strains.

Wardlaw maintained that roughness or smoothness of gram negative organisms is related to the proportion of the cell surface occupied, respectively, by hydrophobic lipid or hydrophilic polysaccharide. This led to his proposal that the substrate or receptor of complement in cell

envelopes is probably the cell wall phospholipid or lipoprotein and that the function of the O-antigen, or lipopolysaccharide, is to make a cell resistant to complement by covering up the surface lipoprotein. An overall scheme for the action of complement, in conjunction with antibody and lysozyme, in immune lysis was proposed as follows: antibody first combines with an O-antigen site. This reduces the overall negative charge on the cell and also provides an anchoring point for complement. Complement then either combines wholly with the lipoprotein adjacent to the antigen site, or it forms a bridge with one end on the antibody and the other end on the cell-wall lipoprotein. The C'3 component may stimulate the complement protein bridge to contract, pulling the phospholipid out of place and thereby dislocating an area of the cell surface. Serum lysozyme would thus be able to penetrate through the dislocated lipoprotein layer to the inner mucopeptide layer (133).

More recently, Rowley has reported (117) that antibody molecules are fixed at the cell surface as they arrive in this area. At the point of antigen-antibody reaction, complement is fixed and activated, leading to a steady shower of later components of complement, one of which ultimately destroys the permeability characteristics of the bacterial membrane, causing leakage and death. According to this concept a bacterium having twice as much lipopolysaccharide as another will fix the first antibody molecules arriving at its surface approximately twice as far from the underlying protoplasmic membrane, and the 2-fold increase in endotoxin thickness could result in an 8-fold decrease in concentration of complement components at the membrane. After a certain thickness of cell wall is reached then the organism should become totally resistant to the action of antibody and complement due to the impossibility of attaining the required concentration of complement components at the bacterial membrane,

even with overlapping sites of antibody influence. Sensitization is thought to result from an opening up of the lipopolysaccharide matrix by breaking (117)



bonds in the lipid A-heptose regions. Once this has been achieved, the specific antibody molecules may be able to penetrate deeper into the lipopolysaccharide structure and activate complement nearer to the cell membrane (117).

The hypothesis that certain of the host reactivities to endotoxins may be mediated or potentiated by the complement system found support in data obtained by Gewurz *et al.* (33), who also noted that lipopolysaccharide preparations potently induced fixation of complement. The component fixed was C'3t (as was noted by Wardlaw). Detoxification resulted in the loss of ability of the lipopolysaccharide to fix complement. This group proposed that loss or alteration of substrates which support the binding or interaction, or both, of the complement system, occurs during the detoxification procedure.

Development of Tolerance

The fact that repeated daily injections of a sublethal dose of bacterial endotoxin results in a progressive decrease in the pyrogenic as well as toxic responses or, "tolerance," within rabbits was first noted by Beeson (7). Freedman (28) successfully transferred tolerance to the pyrogenic effect of endotoxin in rabbits with plasma and serum from tolerant donors; an achievement which had previously evaded several researchers (7,22). The altered response in recipient rabbits was characterized by disappearance of the second rise in fever and by a

reduction in fever index. From his observations Freedman concluded that both tolerance and its transfer are based upon RES function and are independent of antibody (28). Petersdorf and Shulman (100) summarized most of the information which had accrued up to 1963 and concluded that tolerance involves more than enhanced RES clearance of substances which are known to injure the host's tissues. They indicated that nonspecific substances, whether opsonins, inhibitors, or endogenous pyrogen, probably play a major role in its pathogenesis. Individual experiments undertaken by Ruthenburg *et al.* (121) supported this contention.

Watson and Kim, on the other hand, proposed dual immunological mechanisms of pyrogenic tolerance (134). The first would lead to the acquisition of delayed hypersensitivity (see above), which, in turn, would contribute to the intensity of the pyrogenic responses, while the second would bring about induction of a classical antibody which then assists in the destruction of the endotoxin by a normal RES. Further experiments by Freedman (29) resulted in a modification of his earlier position and support was given to a larger role for antibody in the tolerant state. Humoral factors capable of providing cross-protection against heterologous endotoxins were also reported as the basis for tolerance to an increased rate of degradation within the vascular compartment.

In one fairly recent study by Freedman and his co-workers it was noted that hemolysin-forming spleen cells can still increase when sheep red blood cells are given to an endotoxin tolerant animal or when carbon is injected prior to the last of a series of daily lipopolysaccharide

injections (30). The implication here was that antibody-forming cells are not altered in an endotoxin-tolerant animal. One main conclusion formulated by this group was that the failure of tolerant mice to demonstrate an elevation in number of hemolysin-forming spleen cells following endotoxin administration was probably due to inactivation of the endotoxin in tolerant animals, resulting in its absence at sites from which nucleotide (or other) stimulators of plasma cells can be released (30). Recent investigations by Greisman and Woodward (35) produced results which pointed to the existence of 2 distinct mechanisms for the biphasic febrile response to endotoxins, one rapidly acting and extrahepatic, the other slower in onset and hepatic in origin. The findings were said to support the concept of a target role for the Kupffer cell in endotoxin tolerance and they provided the basis for the following 4 principles: (a) extra hepatic mechanisms dominate the first phase of the biphasic febrile response to endotoxin; (b) hepatic mechanisms dominate the second phase of the response; (c) the liver of the tolerant rabbit clears directly perfused endotoxin more efficiently than the liver of the nontolerant animal; and (d) despite the more efficient uptake of directly perfused endotoxin by the liver of the tolerant rabbit, the second phase of fever usually remains markedly inhibited (35). These principles, along with other data obtained, led to the suggestion that tolerance to the pyrogenic activity of bacterial endotoxin is based upon increased uptake of the toxin by hepatic Kupffer cells which have become refractory to the further release of endogenous pyrogen (35).

## Studies on the Lipopolysaccharides of *Pasteurella multocida*

### Extraction of Surface Antigens from *P. multocida*

The few studies of the lipopolysaccharide antigens from *Pasteurella multocida* have contributed little to the total understanding of this organism. But, for whatever reason, a survey of current literature clearly indicates an apparent decrease in studies being carried out on the antigenic structure of this species.

Pirosky (101,102,103,104) was the first to apply the technique of Boivin and Mesrobeanu (14) to the extraction of *Pasteurella* somatic antigens. The glyco-lipid material extracted was thought to be of the same nature as the Boivin preparations. In a series of experiments with extracts from both smooth and rough strains, Pirosky found that his extracts were both antigenic and immunogenic. He obtained antisera for these which equaled those produced against whole bacterial cells in ability to protect mice. The somatic antigen was noted in both smooth and rough bacterial strains, although the rough strains were said to contain twice as much as smooth forms. Cross-precipitation tests indicated that the antigens were serologically distinct. MacLennan and Rondle (71) used the procedure of Westphal *et al.* (136) to extract lipopolysaccharides from *P. multocida* which they found to contain an aldohexose sugar, as well as glucosamine and galactose. It was their opinion that the serological specificity which they had observed could be attributed to the lipopolysaccharide components of the extracts. Four type I (Roberts) strains were reported to possess a common thermostable antigen which was absent from the other types and 5 type III strains also contained a type-specific antigen which was thermostable. The results of this study correlated well with the immunological type specificity

established by Roberts (113) on the basis of his passive protection experiments, although it was not implied that the lipopolysaccharide antigens were necessarily protective.

Bain and Knox used the phenol-water method to extract and purify lipopolysaccharides and noted that only this substance is capable of being absorbed on the surface of erythrocytes (3). Pure polysaccharide and other extracted substances having no heptose content failed to show hemagglutination in antisera against whole cells. In addition to heptose, glucosamine and galactose, these workers found that glucose was also present in the type I (Roberts) strains which they examined. Carter (21) employed a modification of the phenol-water extraction procedure to obtain lipopolysaccharides which were used to develop a standardized hemagglutination procedure for measuring *Pasteurella* antibodies. Endotoxin was extracted from a type E (Carter) strain of *P. multocida* by Perreau and Petit (98), using the phenol-water procedure of Westphal *et al.* The product was reported to be antigenic and to parallel lipopolysaccharide from type B strains in elementary composition, toxicity and serological properties, although serologic techniques revealed qualitative differences between them.

Namioka and Murata (83) used formalinized, boiled and 1 n HCl-treated bacterial cells to produce antisera for a study on *Pasteurella* somatic antigens. Only the 1 n HCl treatment proved to be satisfactory for the serological procedures developed. The results of their work is discussed in the next section of this review. Heddleston *et al.* (40) and Rebers *et al.* (108), using cold, formalinized saline and centrifugation at 105,000 x g, obtained products which were said to be heat-stable, "particulate," lipopolysaccharide-protein antigenic complexes. These "particulate antigens" demonstrated many of the properties ascribed to

endotoxins and mice surviving injection of such preparations developed specific active immunity against homologous bacterial strains. Only cells of the homologous strain were agglutinated by antisera against them, but serological cross reactions between antigens from a bison strain and an avian strain were noted in gel-diffusion plates. In a study by Prince and Smith (105,106,107), the phenol-water procedure was used to extract lipopolysaccharide fractions which were examined. Baxi *et al.* (6) also used this extraction technique to obtain type B and E (Carter) endotoxins for immunodiffusion studies. Their results showed that the "heat stable" antigen from type B cells differed from that of type E strains whereas lipopolysaccharides from both types were very similar, if not identical.

Serological Typing of *P. multocida*

That the existing methods for typing *P. multocida* (19,13) were inadequate was first emphasized strenuously by Bain and Knox (3) when it was noted that Australian and Asian strains of the organism, having the same capsular types, differed in their somatic lipopolysaccharide composition or arrangement. Namioka and Murata (83,84,86), recognizing that the somatic antigens might be important with respect to the virulence of the organism, undertook a series of studies utilizing strains isolated from all the major animal hosts and representing all the known capsular types. By means of cross-absorption tests, O-antigens from *P. multocida* were determined to be of 2 types: common and specific (83). Eleven distinct serological types, based on specific O groups and Carter's capsular types (19) were determined (85,86). Some of these could be further divided into "subtypes" and it was noted that a relationship existed between serological type and host distribution (85). These investigators found that a significant degree of reproducibility could be obtained only if the antigens were treated with 1 n HCl.

Heddleston (39) examined 2 strains of *P. multocida* of avian origin and found that they differed immunogenically and serologically as well as in their pathogenicity for animals. Four distinct serotypes among those *Pasteurella* causing fowl cholera have now been found, based on O antigen differences. All strains pathogenic for bovines, when tested by the Namioka-Murata procedure, were found to contain the O group antigen given the numerical designation "6". All were either of Carter's capsular type "B" or "E". Sato *et al.* (122) examined 21 rabbits suffering from respiratory disease and found that all of them contained O antigen "1" and were uniformly of capsular type "A". This serotype had previously been associated with swine pneumonia (85).

Bacteria contained in 2.5% saline and shaken in a mickle disintegrator until no capsular material could be demonstrated were extracted with phenol-water by Prince and Smith (107). They also used 1 n HCl treated cells for their extensive immunodiffusion studies of both capsular and somatic antigens of *P. multocida*. Sixteen soluble antigens were found which were shared by bovine and avian strains. Two capsular antigens, designated $\alpha$ and $\beta$ by these workers, were found and both appeared to be type-specific in type B and E (Carter) strains although the $\alpha$ antigens were found to have some nontype-specific antigenic determinants which were present in noncapsular components of other types. Saline washed, phenol-extracts contained only a single lipopolysaccharide antigen which was designated $\gamma$ (107). HCl-treated cells were found to contain small amounts of type-specific capsular polysaccharides, indicating that these substances are incompletely removed by the treatment procedure employed by Namioka and Murata (83). At least some of the somatic antigenic specificities observed by Namioka were confirmed by these workers, however, and they concluded that the "$\gamma$" antigen is very probably responsible

for the O somatic types which have been determined. A similar immunodiffusion study by Baxi *et al.* (6) has provided further confirmation of the specificities reported by Namioka and Murata. Type B and E strains were shown by immunoelectrophoresis and gel-diffusion to have different capsular antigens but very similar somatic lipopolysaccharides.

Attempts to type an even greater number of these organisms and further elucidate the relationship between serotype and host distribution and disease (if any) have not yet been made. As yet there have been no attempts to determine the structure and sequence of sugars of the O antigens of *P. multocida* serotypes. Finally, existing correlations between frequency of serotype isolation and pathogenicity for experimental animals are not adequate.

# MATERIALS AND METHODS

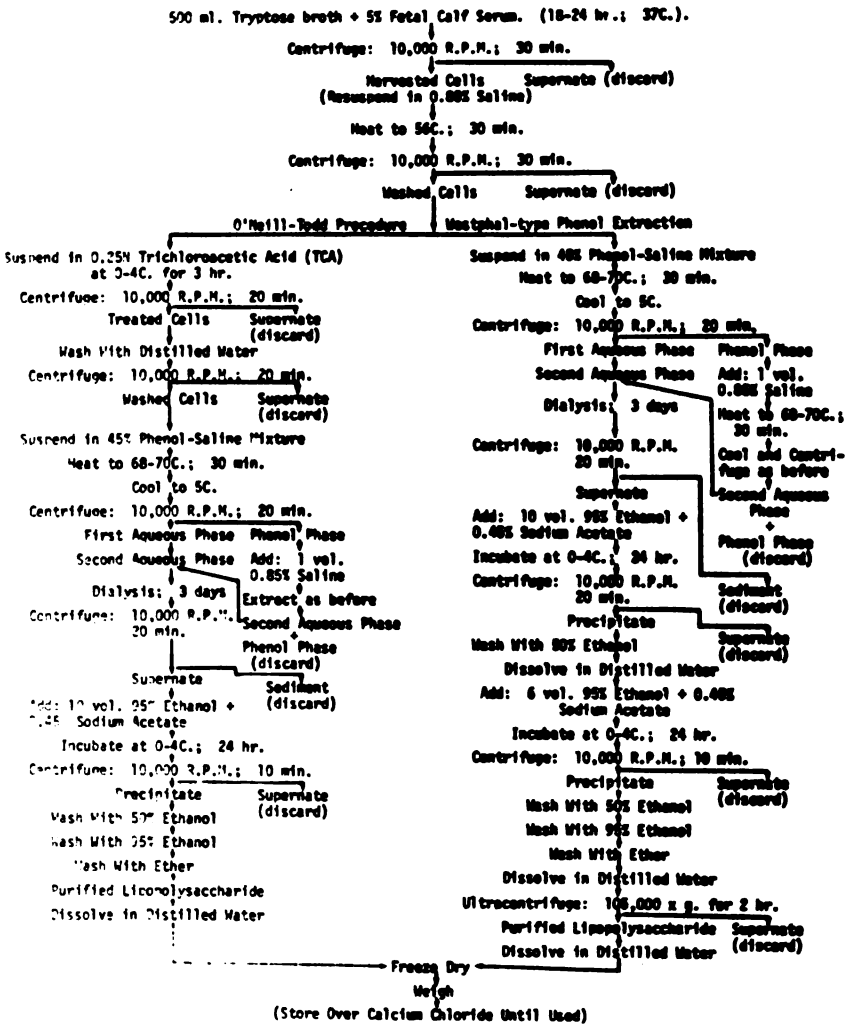
## Cultures

Eleven strains of *P. multocida*, isolated from a variety of animal hosts, were used. Included in the study were representatives of the mucoid, smooth and rough colonial variants. All strains were maintained on Difco Stock Culture Media. Prior to extraction the organisms were subcultured on blood agar at 37 C. for 18 to 24 hours and biochemical tests performed to confirm their identity. Pure subcultures were maintained in tubes of fresh stock culture agar.

## Extraction and Purification of Lipopolysaccharides

All cells were grown in 250 or 500 ml. of tryptose broth (Difco) supplemented with fetal calf serum (Grand Island Biological Co.) which had previously been heated to 56 C. for 30 minutes. Final serum concentration was 5%. Flasks were incubated with mild shaking for 18 to 24 hours at 37 C. in a gyratory water bath. Two different procedures were employed for the extraction and purification of lipopolysaccharides (LPS). The first was similar to the method developed by Westphal *et al.* (136), in which extracted material is fractionated and ultracentrifuged. A second method extended the O'Neill-Todd procedure (90) to include ethanol fractionation of the dialyzed aqueous phase obtained after phenol extraction of trichloroacetic acid (TCA) treated cells. A flow diagram detailing the procedures used is shown (Figure 3).

(Fig. 3) FLOW DIAGRAM OF THE PROCEDURES USED TO EXTRACT AND PURIFY LIPOPOLYSACCHARIDES FROM PASTEURELLA MULTOCIDA



## Evaluation of the Purity of LPS Preparations: Ultraviolet Absorption

Small amounts of the extracted material were suspended in distilled water to a concentration of approximately 1 mg./ml. The absorption of these suspensions at 280 and 260 mμ was determined using a Beckman DB spectrophotometer (Beckman Instruments, Inc.) with recorder (Model-SRL, E. H. Sargent and Co.) attached. A scan of all wavelengths from 300 mμ to 230 mμ was made. Protein and nucleic acid content was determined from a prepared nomograph (prepared by E. Adams, distributed by the California Corporation for Biochemical Research, Los Angeles).

## Immunization of Rabbits

LPS extracts which had been maintained in the freeze-dried state were dissolved in 0.2% formalinized saline and emulsified in an equal volume of Freund's incomplete adjuvant (Difco). White, New Zealand rabbits weighing between 2.5 and 4.0 kg. were bled from the marginal ear vein and the serum collected. Merthiolate was added to a concentration of 0.1% and the serum maintained at 4 C. pending future use. Two rabbits were injected with different amounts of each preparation. One rabbit of each pair received a subcutaneous (SQ) injection of 1.0 ml. of emulsion containing 100 μg. LPS/kg. body weight in each of 2 sites, while the second rabbit was given one-half this amount. Rabbits were bled from the marginal ear vein 2 weeks after the initial LPS injection and approximately 20 ml. of blood was collected from each. A second dose of LPS was then given. Rabbits which had initially received the larger injection were challenged with 200 μg LPS/kg. body weight in each of 2 subcutaneous sites and the second group was challenged with 100 μg LPS/kg. Suspensions for the challenge injections were made in 0.2% formalinized saline; Freund's adjuvant was not used. One week after challenge all rabbits were

bled by cardiac puncture and the serum collected and maintained at 4 C. with 0.1% merthiolate added as a preservative.

Purified LPS from 6 of the strains used for this study was administered to the rabbits in an attempt to produce antibodies. It was thought that emulsion with incomplete adjuvant might aid in the induction of antibody formation by maintaining toxic LPS within the host's system for a longer period than would normally occur. A large challenge dose should then provoke an anamnestic response of sufficient magnitude to obtain antibodies which are detectable in serum tested against endotoxins extracted from homologous organisms.

## Immunodiffusion Studies

Difco Noble agar was used for immunodiffusion studies. This was dissolved (1 gm./100 ml.) in freshly made 0.85% saline, heated to boiling and filtered through a milipore microfiber glass disc prefilter (type AP 20) while hot. The medium was then autoclaved at 121 C. for 15 minutes and merthiolate added to a concentration of 0.01%. Before solidifying the medium was dispensed into 50 x 12 mm. disposable petri plates with tight-fitting lids. Small wells were made in the hardened agar with a distance of approximately 1.0 cm. between each well. Antisera against whole bacterial cells of several of the strains examined are maintained routinely by Dr. G. R. Carter, for use in this researcher's studies, and were readily available. Wells made in the center of diffusion plates were filled with either the anti-whole cell-serum or serum from the rabbits immunized with the purified LPS extracts. All sera were heated for 30 minutes at 56 C. prior to use. Formalinized saline suspensions of the endotoxins were placed in surrounding wells and the plates left at room temperature. All plates were examined for 7 to 10 days for the formation

of antigen-antibody precipitates within the agar. In some instances rapid absorption from the wells occurred and it was necessary to refill them 1 or 2 times.

## Mouse Inoculation

An inbred strain of Webster mice maintained in the Department of Microbiology's Clinical Microbiology Laboratory was used for mouse lethality tests. Serial, 2-fold dilutions of purified LPS were made in formalinized saline and 5 to 10 young mice, varying in age from 1 day to 4 weeks, were injected either intraperitoneally or intracerebrally with 0.1 ml. amounts of each dilution. The range of LPS concentrations was 6.25 to 200 μg. Mice were observed for a period of 2 weeks and all deaths occurring within this time were recorded.

## Induction of the Localized Shwartzman Reaction

Since induction of characteristic Shwartzman-type reactions by endotoxin is often demonstrable in rabbits and is a method of assay used by many workers, it was decided to examine the ability of purified LPS to initiate a local skin response. Because of the small number of rabbits available during this study, animals which had been previously inoculated in the immunization experiment were also used for the skin reaction test. Only 1 rabbit was injected with each of the 7 preparations tested. The endotoxin was administered by intradermal injection of 0.1 ml. of formalinized saline containing 10 μg of LPS. Twenty-four hours later a second injection containing 25 μg. of LPS was administered intravenously in 0.25 ml. of saline. Observations were made for 48 hours after the second injection was given and at the end of this period foci of hemorrhage and dermal necrosis were measured. The area of lesions was calculated as the product (in $cm.^2$) of 2 diameters taken at right angles. Some, but not all,

of the animals were inoculated with endotoxin from the same stock preparation that was used to immunize them in the earlier experiment.

## Inoculation of Chicken Embryos: Preparation of Dilutions

After the purification process, extracted endotoxins were dissolved in 0.2% formalinized saline to a concentration of 1.0 mg./ml. and maintained as stock solutions. Prior to inoculation of the embryos, serial, 5-fold dilutions were made from the stock solution in 0.2% formalinized saline. Equivalent amounts of the diluent were injected into control embryos in all experiments, except where stated otherwise. Asepsis was rigorously maintained during the preparation of dilutions and inoculation of embryos.

## Inoculation Technique

Most embryos used in this study were from White Leghorn chickens although a few embryos from New Hampshire Red hens were of necessity included. In all but 2 experiments embryos were inoculated on the eleventh day of incubation.

The procedure used for chicken embryo inoculation was as follows: A cardboard box (14 in. x 9 in. x 12 in.) was shortened to about one-half its original height, a slit made at one end and a small hole having the approximate shape of an egg cut into its inverted bottom surface. A position-adjustable lamp (Bausch and Lomb, catalog no. 31-33-53) with movable light compartment was fitted in the slit so that its lens rested close to the egg-shaped hole. This construction served as an excellent apparatus for candling and inoculation of embryos. Eggs were candled and a small triangle marked off over a prominent allantoic vein. An electric hand drill fitted with 2 abrasive discs was used to cut through the shell, forming the triangular windows. After the membranes were exposed by removal of shell fragments, embryos were reincubated for at least 1 hour

to allow fixation of the vein as performed by Finkelstein (27).

Disposable tuberculin syringes (1 cc.) fitted with 27-gauge disposable needles were used for intravenous inoculation of the embryos. Separate syringes were aseptically filled with each LPS dilution. Embryos were placed in the egg-shaped perforation and candled while being inoculated. For most experiments, 10 to 15 embryos were inoculated with each dilution; however, due to a severe fertility problem among the eggs obtained at the beginning of this study, it was sometimes necessary to limit this number to as few as 5 embryos per dilution. Endotoxin was injected intravenously in 0.05 cc. amounts and injection was made in the direction of the flow of blood.

A small amount of bleeding to the outside of the embryo was normal and was not harmful to the embryo. A study of embryos which showed internal hemorrhage after inoculation revealed that in about 50% of the instances wherein normally nontoxic dilutions or control medium were used, death resulted in about 24 hours. Subsequently, all embryos in which internal hemorrhage was observed within 5 hours postinjection of endotoxin were discarded and not included in the final results.

In the early experiments, a drop of sterile mineral oil was used to clear the shell membrane as described by Smith and Thomas (126). It was observed, however, that a larger percentage of embryos appeared to hemorrhage internally when this substance was used than did so without it.

To determine if this compound might be responsible for a detrimental effect on the embryos 2 drops of sterile mineral oil were placed either on the chorioallantoic membrane (CAM), the exposed vein or the intact shell membrane. No hemorrhage or death occurred in any of the embryos receiving this treatment. Although no correlation between application of mineral oil and death of embryos could be verified, the practice was

discontinued with no resultant increase in difficulty of inoculation. When embryos showing internal hemorrhage were discarded, death among controls injected with 0.05 ml. of formalinized saline was well below 5%. It was noted from observation of control embryos that covering the membranes was unnecessary.

In most instances death occurred within a few hours, although in embryos inoculated with the lower LPS dilutions movement could sometimes be observed at 14 to 18 hours postinoculation, before death finally occurred. Results were recorded 24 hours after inoculation. Routine sterility tests were performed on representative embryos at 5 days after their inoculation. Sterile cotton swabs were used to obtain samples of allantoic fluid and inoculation was made onto blood-agar plates and into tubes of semisolid Brain Heart Infusion broth. There were no instances of growth from these inoculations. Results were recorded as the number of deaths in 24 hours over the number of embryos injected. The 50% lethal dose ($LD_{50}$ for chicken embryos, or $CELD_{50}$) for each LPS used was estimated from a graph by the probit method as illustrated by Batson (4).

## Inoculation of Whole Bacterial Cell Suspensions

Five *P. multocida* strains were incubated for 18 to 24 hours at 37 C. in the medium described above (see extraction of LPS) and then harvested by centrifugation. The cells were washed once in physiological saline, centrifuged and resuspended in formalinized saline. The Wright method was used to obtain an approximation of the number of bacterial cells in the (stock) suspensions. All but 1 contained approximately $10^8$ bacteria/ml. Ten-fold dilution of each stock preparation was made from which serial, 5-fold dilutions were subsequently prepared for chick embryo inoculation. Four or five different dilutions were administered to 11-day-old chick

embryos as described above. Although most susceptible embryos died within 24 hours, observations were made for 48 hours. Fifty percent lethal doses were determined as before.

## Statistical Evaluation of Results

The significance of all embryo-inoculation results was determined by the use of chi-square $X^2$ contingency tables as recommended by Batson (4). This method effectively detects differences in observed occurrences from those which would be expected by chance. The significances of individual differences among the results of several tests of the same extract, and differences in the various extracts were determined by use of the Brandt-Snedecor formula (4).

# RESULTS

The cultures examined in this study were not fresh isolates. Several strains had been studied previously and had been maintained for several years on stock culture agar. Various changes have been observed by a number of workers in cultures maintained under these conditions. It was therefore necessary to examine each strain with respect to its biochemical reactions and colonial properties. The biochemical reactions of strains pertinent to this work are presented in Table 1. No significant dissociation of strains from the form originally observed was noted. Only one strain failed to produce appreciable indol in 24 hours. Several strains were slow to ferment mannitol and sucrose while most fermented lactose and maltose to some extent after 36 to 48 hours. In addition, 3 strains produced very weak oxidase reactions. Colonies showed a negative reaction to this reagent after 2 minutes and weak reactions after 3 or 4 minutes. These results are not inconsistent with reactions exhibited by many members of this species, however, and all cultures were considered representative of the species *P. multocida*.

Table 2 lists the strains employed in this project with respect to their animal origin, the associated disease or condition and serological classification, based on the serotyping procedure of Namioka and Murata (83). Antisera specific for all of the Namioka-Murata immunotypes was not readily available and no attempt was made to produce and absorb antisera for the purpose of classifying strains of unknown immunotype. It is felt that such a procedure may be extremely valuable if future

Table 1. Biochemical reactions of the *P. multocida* strains used in this study

| Strain: | Growth on MacConkey | Acid from Glucose | Lactose | Maltose | Mannitol | Sucrose | Indol | Methyl-Red | Nitrate | Oxidase | Urease |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ATR-751 | -[a] | +[b] | - | - | + | + | + | - | + | + | - |
| L-611 | - | + | ±[c] | ± | ± | + | - | - | + | +[d] | - |
| 3397 | - | + | - | - | + | + | + | - | + | + | - |
| 63-875 | - | + | ± | ± | + | + | + | - | + | + | - |
| B-19 | - | + | ± | ± | + | + | + | - | + | +[d] | - |
| VA-3 | - | + | - | - | ± | ± | + | - | + | + | - |
| P-1059 | - | + | ± | ± | + | + | + | - | + | + | - |
| P-1234 | - | + | ± | ± | + | + | + | - | + | + | - |
| B-1046 | - | + | ± | ± | + | + | + | - | + | +[d] | - |
| B-1048 | - | + | ± | ± | + | + | + | - | + | + | - |
| 656 | - | + | ± | ± | + | + | + | - | + | + | - |

[a]Negative; [b]Positive; [c]pH indicator showed that some acid was produced in carbohydrate after 36 hours; [d]Very weak oxidase reaction; usually evident only after 2 to 5 minutes.

Table 2. Animal source, associated disease and serotype of the various *P. multocida* strains

| Strain | Source | Associated disease | Serotype[a] |
|---|---|---|---|
| ATR-751 | Primate | Peritonitis | Not known |
| L-611 | Canine | Not known | Not known |
| 3397 | Swine | Pneumonia | 1:A |
| 63-875 | Avian | Fowl cholera | Not known |
| B-19 | Avian | Fowl cholera | Not known |
| VA-3 | Avian | Fowl cholera | 5:A |
| P-1059 | Avian | Fowl cholera | 8:A |
| P-1234 | Bovine | Hemorrhagic septicemia | -:E |
| B-1046 | Bovine | | Not known |
| B-1048 | Bovine | | Not known |
| 656 | Bison | Hemorrhagic septicemia | 6:B |

[a]Based on the serological groupings proposed by Namioka and Murata (81).

studies along this line are pursued. The present study, however, was intentionally designed to examine only the general relatedness between endotoxins from strains of different animal origin (see Discussion).

## Ultraviolet Absorption

The ultraviolet absorption of purified LPS preparations was examined by scanning wavelengths between 230 mμ and 300 mμ. Absorbancies were recorded onto graphs and the values noted at 280 mμ and 260 mμ were used to determine concentrations of protein and nucleic acid, respectively. The results are shown in Table 3. It will be noted that for both methods employed the rigorous purification process resulted in products containing negligible amounts of these substances. With only a few exceptions there were no peaks of absorbance recorded over the wavelengths scanned.

## Removal of Capsules

Mucoid strains of *P. multocida* were subcultured up to 10 times on blood or tryptose agar in an effort to obtain nonmucoid variants. Colonies demonstrating slight, or no, mucoidness were selected and these were used for the extraction of LPS. All strains were washed for a considerable length of time in saline before extraction and ethanol fractionation was carried out later in the process. Although absolute removal of nonendotoxic polysaccharides was not considered likely, it was anticipated that only trace amounts would remain in the purified extract.

## Immunization Results

One characteristic of lipopolysaccharides which has been reported many times in the past is the poor immunogenicity of these substances. Specific antibodies have rarely been demonstrated after immunization with

Table 3. Protein and nucleic acid content in extracted lipopolysaccharides

| Extract From[a] | Method of Extraction | Absorbance 280 nm. | Absorbance 260 nm. | Percent contaminants[e] (mg.) Protein | Percent contaminants[e] (mg.) Nucleic acid |
|---|---|---|---|---|---|
| ATR-751 | Phenol-water[c] | 0.075 | 0.075 | 0.060 | 0.002 |
| L-611 | Phenol-water | 0.014 | 0.020 | 0.010 | 0.0006 |
| 3397 | Phenol-water | 0.030 | 0.017 | 0.040 | 0.00 |
| 63-875 | Phenol-water | 0.011 | 0.012 | 0.003 | 0.0004 |
| B-19 | Phenol-water | 0.017 | 0.008 | 0.020 | 0.00 |
| VA-3 | TCA-Phenol[d] | 0.032 | 0.030 | 0.025 | 0.001 |
| P-1059 | TCA-Phenol | 0.014 | 0.00 | 0.020 | 0.00 |
| P-1234 | TCA-Phenol | 0.008 | 0.010 | 0.003 | 0.0004 |
| B-1046 | TCA-Phenol | 0.012 | 0.012 | 0.003 | 0.0004 |
| B-1048 | Phenol-water | 0.003 | 0.004 | 0.001 | 0.0001 |
| 656 | TCA-Phenol | 0.010 | 0.00 | 0.010 | 0.00 |
| 0126:B16[b] | TCA-Phenol | 0.051 | 0.083 | 0.020 | 0.002 |

[a]Strain of *P. multocida* from which the purified LPS was obtained.

[b]*E. coli*.

[c]Modification of the Westphal extraction procedure (see Materials and Methods).

[d]Method of O'Neill and Todd (see Materials and Methods).

[e]Interpreted from a nomograph prepared by E. Adams (see Materials and Methods).

protein-free LPS. With these reports in mind, an attempt was made to elicit the production of antibodies against the materials extracted for the present study. Two rabbits were immunized with each of 6 different extracts as reported in Materials and Methods. No precipitates were formed in immunodiffusion plates between any of the purified endotoxins and their "homologous" serum. These results suggest that there was insufficient protein complexed with the molecules to evoke active synthesis of specific antibody. The possibility cannot be discounted that antibodies may have been produced but in quantities too small to be detected by the systems employed. In any event, the absence of a detectable, specific immune response supports a poorly-immunogenic, protein-free, lipopolysaccharide character for the preparations extracted.

## Immunodiffusion Studies

The reactions noted in gel between the extracts and antisera prepared against whole bacterial cells provide even greater support for the LPS nature of these substances. It can be seen in Table 4 that several of the purified extracts formed precipitates with one or more of the anti-whole cell sera. Most of the lines of precipitation were located in very close proximity to the antigen well corresponding with the normal location of precipitates formed by lipopolysaccharides. Strains P-1059 and 3397 reacted with several of the antisera suggesting that common somatic antigens, or antigenic determinants, are shared by at least some of the members of this species. Another interesting possibility results from the finding of Prince and Smith (107) that "α" antigens present in the capsules of bovine and avian strains which they examined are extremely difficult to completely separate from the cell wall. These components were found to be antigenically similar to somatic "γ" antigens and to

Table 4. Reaction of purified lipopolysaccharides with anti-whole cell *P. multocida* serum in immunodiffusion plates

| Antigen (LPS from) | Antisera Reacted with | Precipitate | |
|---|---|---|---|
| | | Proximity to antigen well | Comments |
| P-1059 | P-1059, VA-3, 989, CD-90A | Very close; bordering antigen well with some sera | Single distinct line |
| 3397 | P-1059, VA-3, 989, CD-90A | Bordering or very close | Single band; not very distinct |
| B-1048 | 989 | Approx. 1/4 distance toward antiserum well | Very broad band. Appeared to represent a single antigen |
| CD-90A | CD-90A | Bordering | Single line; small but distinct |
| L-611 | VA-3 | Close | Board; indistinct band |
| 656 | 989 | Very close | Single small indistinct band |

possess type-specific antigenic determinants in addition to some nontype-specific determinants which were shared by noncapsular components of other types.

## Yield of LPS

The extraction procedures employed by this worker yielded very low amounts of purified LPS, with the average range lying between 2 and 4 mg. of LPS per 500 ml. of cells extracted. A relatively low percentage yield was anticipated since essentially complete removal of all contaminants was the purpose of the adopted methods. However, it was expected that somewhat more than 4 mg. of purified toxin would be obtained from the cells grown in 500 ml. of medium.

## Mouse Lethality Tests

Injection of mice with the quantities of LPS already reported (Materials and Methods) resulted in an insufficient number of deaths to obtain $LD_{50}$ values. Less than 50% of the mice were killed when 200 μg. of endotoxin was injected. From these observations it became evident that the quantities of LPS which would be required to adequately determine and compare lethalities of these substances for mice would severely limit in number other experiments more closely related to the proposed purpose of this study. Hence, investigations along this line were discontinued, and no data regarding mouse lethality is tabulated in this report.

## Induction of Shwartzman Reactions

Table 5 shows the results of an attempt to induce localized Shwartzman reactions in rabbits. Three strains failed to produce noticeable skin reactions. There appear to be several possibilities that might explain the inconsistent results; however, since the number of animals available

Table 5. Localized Shwartzman reactions resulting from *P. multocida* lipopolysaccharide injection into rabbits

| Extract from | Shwartzman Reaction ($cm.^2$)[a] | Comments |
|---|---|---|
| P-1059 | 3.60 | Sizeable necrotic area, characteristic reaction |
| B-1048 | 1.65 | Necrotic destruction slight |
| P-1234 | 1.00 | Small area of hemorrhage |
| 656 | N.R.[b] | Several petechiae, no basis for measurement |
| 3397 | 1.60 | Marked hemorrhagic necrosis, very characteristic reaction |
| 63-875 | N.R. | |
| L-611 | N.R. | |

[a]Area - in sq. cm. - calculated as the product of 2 diameters measured at right angles.

[b]No reaction.

for testing was limited and only one was injected with each preparation, any attempt to explain such inadequate data would be speculatory. In addition to the possibility that insufficient LPS to elicit a reaction may have been injected, one might also consider such proposed influences as prior exposure to related organisms (123) or to a protein component (31), and the induction of hypersensitivity (61).

## Toxicity of *P. multocida* for Chicken Embryos

In Table 6, data pertaining to the chicken embryo inoculation experiments, which constitute the foundation of this thesis, is summarized and its significance indicated. Fifty percent lethal doses differed 125-fold between the lowest and highest values obtained. It will be observed, however, that in every experiment recorded, the results were significantly different from any result which would be expected due to random chance occurrence. (In all but one experiment P was less than 0.001.) No significant differences were detectable between the toxicities observed when 2 or more experiments were performed using the same extract. These evaluations confirm the reliability of the data obtained and the reproducibility of results.

## Statistical Analysis of Toxicities

Since the main purpose of this investigation was to compare the toxicity of endotoxins on the basis of their animal source, at least 2 strains were selected which were isolated from each animal species represented in Table 6. The first extractions, however, were made on the growth from 250 ml. of broth, and because of the low endotoxin yield mentioned above, many of the products were not of sufficient quantity to be tested. In several instances the total yield was less than 1.0 mg. of purified LPS and this was not thought to be an adequate amount to work

Table 6. Compiled data: Embryos inoculated, median lethal doses and significance of differences within groups

| Animal Source | Extract from | Embryos:no. died/no. inoculated[a] 0.10 (µg.) | 0.02 (µg.) | 0.004 (µg.) | 0.0008 (µg.) | $CELD_{50}$ (µg.)[b] | Controls Injected[c] | Controls Died | Significance of Differences[d] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Avian | 63-875 | 8/11 | 3/11 | 0/10 | 0/12 | 0.0580 | 5 | 0 | P < 0.001 | N.S.[g] | P < 0.001 |
| | 63-875 | 14/14 | 6/15 | 0/15 | 0/15 | 0.0250 | 14 | 0 | P < 0.001 | | |
| | VA-3 | 5/5 | 2/5 | 2/5 | 0/6 | 0.0094 | 5 | 0 | P < 0.001[f] | - | |
| | VA-3 | 9/9 | 5/10 | 3/9 | 0/9 | 0.0089 | 7 | 0 | | | |
| | B-19 | 5/5 | 6/6 | 1/5 | 1/5 | 0.0063 | 0 | 0 | P < 0.001 | - | |
| | B-19 | 10/10 | 7/10 | 1/12 | 0/8 | 0.0082 | 11 | 0 | | | |
| | P-1059 | 12/12 | 10/10 | 8/12 | 1/11 | 0.0030 | 0 | 0 | P < 0.001 | N.S. | |
| | P-1059 | 9/9 | 18/18 | 16/18 | 3/8 | 0.00086 | 0 | 0 | P < 0.001 | | |
| | P-1059 | 13/13 | 13/13 | 10/12 | 0/12 | 0.0028 | 5 | 0 | P < 0.001 | | |
| | P-1059 | 8/8 | 11/11 | 12/12 | 2/12 | 0.00095 | 4 | 0 | P < 0.001 | | |
| Bovine | B-1046 | 9/9 | 8/8 | 8/8 | 2/10 | 0.00094 | 10 | 2 | P < 0.001 | N.S. | N.S. |
| | B-1046 | - | 4/4 | 3/5 | 1/5 | 0.0026 | 0 | 0 | | | |
| | B-1046 | - | 5/5 | 5/6 | 0/5 | 0.0024 | 6 | 0 | P < 0.001 | | |
| | B-1048 | 15/15 | 14/14 | 8/13 | 0/15 | 0.0037 | 0 | 0 | P < 0.001 | N.S. | |
| | B-1048 | 13/13 | 14/15 | 11/15 | 0/15 | 0.0035 | 8 | 0 | P < 0.001 | | |
| | P-1234 | 11/11 | 11/11 | 7/11 | 1/12 | 0.0031 | 17 | 0 | P < 0.001 | N.S. | |
| | P-1234 | 10/10 | 6/6 | 1/9 | 0/7 | 0.0060 | 0 | 0 | P < 0.001 | | |
| | P-1234 | 12/12 | 9/9 | 12/12 | 1/11 | 0.0010 | 7 | 1 | P < 0.001 | | |
| Bison | 656 | 8/8 | 9/9 | 4/9 | 3/10 | 0.0027 | 5 | 1 | P = 0.001 | N.S. | |
| | 656 | 10/10 | 14/14 | 5/10 | 0/9 | 0.0040 | 8 | 0 | P < 0.001 | | |

Table 6 (cont'd.)

| Animal Source | Extract from | Embryos:no. died/no. inoculated[a] 0.10 (µg.) | 0.02 (µg.) | 0.004 (µg.) | 0.0008 (µg.) | $CELD_{50}$ (µg.)[b] | Controls Injected[c] | Died | Significance of Differences[d] | |
|---|---|---|---|---|---|---|---|---|---|---|
| Swine | 3397 | 5/5 | 4/5 | 2/4 | 0/4 | 0.0051 | 4 | 0 | P < 0.001 | N.S. |
| | 3397 | 6/6 | 5/5 | 2/4 | 0/4 | 0.0040 | 5 | 0 | | |
| | 3397 | 10/10 | 8/10 | 3/9 | 1/9 | 0.0054 | 0 | 0 | P < 0.001 | |
| | 3397 | 9/9 | 10/10 | 3/11 | 0/11 | 0.0052 | 11 | 1 | P < 0.001 | |
| Canine | L-611 | 4/8 | 1/10 | 0/7 | 0/6 | 0.0870 | 4 | 0 | P < 0.05 | N.S. |
| | L-611 | 8/14 | 2/16 | 0/14 | 0/16 | 0.1020 | 14 | 2 | P < 0.001 | |
| Primate | ATR-751 | 6/6 | 6/6 | 4/7 | - | 0.0035 | 0 | 0 | P < 0.001 | - |
| | ATR-751 | 9/9 | 10/10 | 7/13 | 0/13 | 0.0039 | 8 | 0 | | |
| | 0126:B16[e] | 11/12 | 11/12 | 8/12 | 2/11 | 0.0024 | 6 | 0 | P < 0.001 | N.S. |
| | 0126:B16 | 9/9 | 7/7 | 3/7 | 0/6 | 0.0043 | 7 | 0 | P < 0.001 | |

[a] Embryos in which hemorrhage was seen are not included in this number.

[b] Estimated by the probit method of Miller and Tainter (4).

[c] Inoculated with 0.05 ml. formalinized saline.

[d] Calculated by the $X^2$ contingency method (see Materials and Methods).

[e] *E. coli*.

[f] Combination of 2 experiments. Data in this column represents the significance of difference of observed results from those which would be expected due to chance.

[g] Not significant. Column indicates variation in lethality of extracts as experiments are repeated.

with and obtain results which could be considered reliable. Because of the time and labor required to carry out an extraction as outlined in this report, it was not practicable (within the limitations of this project) to repeat this process for the purpose of increasing the effectiveness of comparisons. An adequate amount of LPS was obtained from several strains of bovine and avian origin.

Statistical analysis showed that none of the bovine strains differed significantly with respect to the toxicity of their somatic antigens for chicken embryos. Among the avian strains, however, highly significant differences were observed. Table 7 shows the results of statistical comparison of lipopolysaccharides from avian strains. The extract from strain P-1059 differed significantly from those of other avian strains, while a closer relationship was noted between the rest. The method of extraction apparently made little difference. In fact, a comparison of the mean toxicities of extracts obtained by the different methods showed that the TCA-phenol extracted LPS was only slightly more toxic than that extracted by the phenol-water procedure. Since strain P-1059 represents the most frequently isolated serotype found among avian strains, the possibility that its greater toxicity might reflect its more frequent occurrence in nature was considered. Plate agglutination experiments showed that cells of strain 63-875 (the least toxic avian strain) were agglutinated by antiserum against strain P-1059. Although a reaction with only the capsular antigens of this strain was not excluded as an explanation, it is obvious that until the immunotype of 63-875 is completely determined, no valid correlation can be made between the prevalence of strain P-1059 (serotype 8:A) and the toxicity of its LPS.

In Table 8 are shown the results of statistical comparisons between lipopolysaccharides from strains having different animal origins. Several

Table 7. Statistical comparison of differences in lethality among lipopolysaccharides extracted from avian *P. multocida*

| Extracts Compared | $X^2$[a] | Significance of Difference |
|---|---|---|
| 63-875 vs. 63-875 | 0.81 | N.S.[c] |
| 63-875[b] vs. VA-3 | 4.11 | N.S. |
| 63-875 vs. B-19 | 7.49 | P = 0.05 |
| 63-875 vs. P-1059 | 63.13 | P << 0.001 |
| VA-3 vs. B-19 | 0.45 | N.S. |
| VA-3 vs. P-1059 | 21.94 | P < 0.001 |
| B-19 vs. P-1059 | 16.16 | P < 0.01 |
| P-1059 vs. P-1059 | 3.83 | N.S. |
| All extracts | 68.78[d] | P << 0.001 |

[a]Test statistic; the Brandt-Snedecor formula was applied in order to partition off total $X^2$'s into individual comparisons (4).

[b]If no significant difference existed between individual toxicities of an extract, all experiments done with that extract were combined and comparisons made.

[c]Not significant.

[d]Total $X^2$.

Table 8. Statistical comparison of differences in the toxicity of purified *P. multocida* lipopolysaccharides for chicken embryos, based on the animal source of the organism

| Extracts Compared (animal source) | $X^2$[b] | Significance of Difference |
|---|---|---|
| Total avian vs. total bovine[a] | 8.67 | $P < 0.01$ |
| Avian vs. swine | 64.42 | $P << 0.001$ |
| Avian vs. canine | 111.16 | $P << 0.001$ |
| Avian vs. primate | 66.42 | $P << 0.001$ |
| Avian vs. *E. coli* | 67.65 | $P << 0.001$ |
| Bovine vs. swine | 2.61 | N.S.[c] |
| Bovine vs. canine | 76.21 | $P << 0.001$ |
| Bovine vs. primate | 0.31 | N.S. |
| Bovine vs. *E. coli* | 0.27 | N.S. |
| Swine vs. canine | 37.65 | $P < 0.001$ |
| Swine vs. primate | 0.84 | N.S. |
| Swine vs. *E. coli* | 1.36 | N.S. |
| Canine vs. primate | 39.00 | $P < 0.001$ |
| Canine vs. *E. coli* | 44.35 | $P < 0.001$ |
| Primate vs. *E. coli* | 0 | N.S. |

[a]All experiments with strains having the same animal source were pooled for the comparisons reported.

[b]Test statistic; see a under Table 7.

[c]Not significant.

highly significant differences can be observed. Comparisons reported between avian and bovine strains were made by pooling the data from all experiments with each strain and testing it as a single observation. Although the resultant $X^2$ is highly significant its size was decreased by pooling the data among the avian strains. P-1059 was significantly more toxic than all of the bovine strains which were, in turn, more toxic than the other avian strains.

Although the total $X^2$ resulting when *P. multocida* from other animal hosts were evaluated against the entire avian group was very large for each case, individual comparisons showed: (a) P-1059 LPS was significantly more toxic but that from 63-875 less toxic than LPS from 3397, while LPS from VA-3 and B-19 did not differ significantly from 3397; (b) LPS from the canine strain (L-611) was significantly less toxic than those from all avian *Pasteurella* except 63-875; (c) endotoxin from 2 avian *Pasteurella* was less toxic than the material isolated from a primate strain, while for 2 other strains there was no significant difference; (d) the toxicity of LPS from the *E. coli* strain was identical to that from the primate strain and identical results were obtained when this preparation was evaluated against extracts from avian strains.

Two *P. multocida* strains of canine origin were selected for use in this study. Both produced the so-called "blue" colonial variants, with no demonstrable capsule. After extraction only a minute amount of LPS was recovered from one of the strains. A group of embryos was inoculated with some of this material in an attempt to obtain results referable to those recorded for the second LPS (L-611). However, the $CELD_{50}$ for this experiment was much greater than 0.10 μg., so the data were discarded. Lipopolysaccharide from the canine strain L-611 was the least toxic material examined in this study. Its toxicity was of a significantly

lower order than the toxicity of any other preparation except 63-875. Bovine, swine, primate strains and *E. coli* did not differ significantly from one another.

The experiments summarized in Table 9 were added because of their potential importance, after the main studies had been completed. The cells were not treated extensively and it is therefore not possible to declare that all capsular substance was removed. No correlation of the data obtained from these experiments could be made with the data from chicken embryo inoculations.

Table 9. Toxicity of whole *P. multocida* cells for chicken embryos

| Strain | No. of embryos dying/No. inoculated per dilution | | | | | | | $CELD_{50}$ (cells/ml.)[a] |
|---|---|---|---|---|---|---|---|---|
| | Undiluted | 1:10 | 1:50 | 1:250 | 1:1250 | 1:6250 | Control | |
| 3397 | - | - | 9/9 | 9/9 | 5/11 | 1/7 | 0/6 | $5.3 \times 10^3$ |
| P-1059 | - | 5/5 | 10/10 | 6/11 | 2/10 | 1/10 | 0/5 | $9.0 \times 10^3$ |
| P-1234 | 10/10 | 10/11 | 5/10 | 0/11 | 0/7 | - | 0/6 | $4.3 \times 10^4$ |
| 656 | - | 9/9 | 11/11 | 8/10 | 2/10 | 0/9 | 0/11 | $8.8 \times 10^3$ |
| ATR-751 | - | 10/10 | 12/12 | 1/11 | 0/11 | 0/11 | 0/6 | $5.8 \times 10^4$ |

[a]Estimated by the probit method of Miller and Tainter (4).

# DISCUSSION

This study was aimed at utilizing the acute sensitivity to endotoxin of intravenously injected chicken embryos to detect differences, if any, between *P. multocida* strains isolated from different animal sources. A 125-fold variation in the estimated $CELD_{50}$ was noted and some strains differed extensively in their toxic properties from others. However, for several groups, separated on the basis of their animal origin, no statistically significant differences could be detected.

A considerable body of evidence points to the difficulty in determining distinct differences between the members of the species investigated here: MacLennan and Rondle (71) extracted a lipopolysaccharide with which they were able to demonstrate a single antigenic component. Only one line of precipitation was formed in gel when the LPS was reacted against its homologous antiserum; however, it was necessary to remove or reference (by the formation of lines of identity) nontype-specific antigens to identify this component in whole cells. Bain and Knox (3) commented that the antigenic complexity of *P. multocida* has always been underlined by the numerous cross reactions which may be noted in all attempts to type the organism. Carter and Bain (20) asserted that the difficulty in evolving a satisfactory serological classification of *P. multocida* was mainly due to the remarkable capacity for variation, the close antigenic relationship of strains and the frequent occurrence of strains among animals commonly employed for the production of immune sera (20). Namioka and Murata (83) stated that the pathogenicity of

*P. multocida* varies with each culture. These workers also implied that the capsule of this organism has an influence upon its virulence and they were able to divide its O antigens into 2 components, common and specific antigens. Heddleston (41) reported a high degree of cross-reaction between "particulate" antigens isolated from a virulent bovine and an avirulent avian strain. He postulated that the cross-reactions might result from the presence of antibodies directed against a common component which is present in both preparations. A capsular component designated "$\alpha$-antigen" was found by Prince and Smith to possess some antigenic similarities to the somatic, or "$\gamma$" antigen (107). The capsular $\alpha$ antigen was further stated to be a complex which was type-specific in Carter's type B and E strains but also in possession of some nontype-specific antigenic determinants which were shared with noncapsular components of other types. These researchers successfully induced cross-stimulation of capsular antibody production within bovine and avian strains and between the two. Thus, it was suggested that the $\alpha$-complex probably contains a common protective protein fraction whose ability to protect is dependent upon its availability in individual strains.

The variety of observations (and speculations) summarized above make specific proposals on the basis of the incomplete data presented in this report unsupportable. The added support which would be provided by data relating to several strains from each animal host becomes obvious when the lack of relatedness found within the avian strains is considered. Whether incomplete removal of capsular substance may account for a portion of the differences cannot be definitely stated; however, the author is inclined to discount this possibility, due to the close morphological similarity of all capsulated strains at the start of the extraction process. It is interesting to note that strains P-1059 and 3397 cross-reacted

with several antisera in the immunodiffusion tests. These results have been observed many times with whole cells and LPS from these strains, sometimes after 3 or more rigorous saline washes had been performed. There is no "enlightened" explanation for this at the present time; however, one can perceive the extreme difficulty which will be encountered in trying to apply to such strains criteria which would indicate a specific animal-host relatedness. Further tests using more strains from each animal source would be necessary before a meaningful evaluation could be made.

As indicated above, there is some evidence that several somatic or capsular fractions (e.g., the $\alpha$, $\beta$ and $\gamma$ antigens of Prince and Smith) may participate to a varying extent in the toxicity of *P. multocida*. This suggests a need to isolate and purify the various surface and external components and to test each for toxicity. Further characterization of these fractions, once purified, would also be of great value. One other procedure which would appear to be in order if subsequent studies are conducted is the testing of embryos for antibodies against *P. multocida*. Such tests were not performed during these studies; however, the presence of *P. multocida* antibody, no matter how unlikely, should be determined because of the information a positive result could later provide in respect to the comparative toxicities of different serotypes.

## SUMMARY

In summary, lipopolysaccharides were extracted and purified by 2 different procedures. Eleven strains of *Pasteurella multocida* were used for the study. The antigens were not immunogenic in rabbits and only about half of those tested evoked a localized Shwartzman reaction. Preparations containing up to 200 μg. of lipopolysaccharide demonstrated little toxicity for young mice. A 125-fold difference in the lethality of toxic extracts injected intravenously into 11-day-old chicken embryos was found. Although some significant differences existed, no definite correlation could be made between toxicity for chicken embryos and animal source or prevalence of the limited number of strains examined in this study. Toxicity of whole bacterial cells injected into 11-day-old embryos could not be directly correlated with toxicity of their purified lipopolysaccharides.

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