

INDOLIC COMPOUNDS IN TISSUES OF MICE AND RABBITS
INFECTED WITH PASTEURELLA MULTOCIDA
AND PASTEURELLA HEMOLYTICA

by

MUHAMMAD ZAIYANU ABDULLAHI

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A THESIS

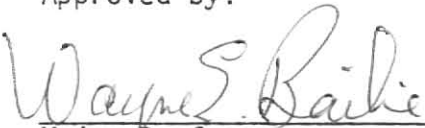
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Major Professor

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INTRODUCTION

Bovine respiratory disease is of great importance to the livestock industry. In respect to morbidity and mortality it ranks second behind diseases which affect the newborn and greatest losses are incurred by the feeder-cattle industry (Lillie, 1974 and McKercher, 1978). Although it may also be recovered as a part of the normal flora of the upper respiratory tract, Pasteurella multocida is also commonly recovered from lung tissues of cattle which have died of this condition (Jensen et al., 1976).

The etiology of this disease is complex and probably involves several species of bacteria as well as viruses, but most investigators consider that this microorganism is associated with the disease either as a primary or secondary agent. The microorganism may also cause disease processes either of an acute septicemic or chronic nature in a variety of other animals and man. In spite of the economic and public health significance of P. multocida infections, the pathogenic mechanism by which it produces disease are poorly understood (Carter, 1975; Carter, 1976; Bruner and Gillespie, 1973 and Merchant and Parker, 1966).

This microorganism when grown in the laboratory in the presence of the amino acid tryptophan, produces the compound indole which is utilized as a differential test for its identification (Cowan, 1974 and Lennette et al., 1980). Indole and the closely related compound, 3-methylindole (3 MI) are lipophilic and have been associated with certain diseases. Both will produce erythrocyte hemolysis and arthritis. Three-methylindole has been associated as an agent in the production of acute bovine pulmonary emphysema (Nakoneczna et al., 1969; Forbes and Neale, 1935; Rogers, 1969;

Yokoyama et al., 1975; Carlson, et al., 1969 and Bradley et al., 1978).

Since indole and 3 MI had been demonstrated as pathogenic mechanisms, it was apparent that if *P. multocida* produced indole during an active infection, this compound might produce tissue damage. References in regard to in vivo production of indole by P. multocida were not found.

This study was designed to determine if indole and/or 3 MI were produced in mice and rabbits during P. multocida infections.

LITERATURE REVIEW

Respiratory disease is a major concern in the livestock industry since 40-80% of all cattle diseases involve this system (Lillie, 1974). Many investigators have associated Pasteurella multocida with respiratory syndromes either as a primary or secondary cause (Jensen et al., 1976). Although this microorganism is a part of the normal flora of the upper respiratory tract of man, animals and birds, it is considered as the causative agent of hemorrhagic septicemia and pneumonia of cattle throughout the world. Pasteurella multocida is one of the etiologic agents of pneumonia of swine, sheep and goats, rabbits septicemia, and mastitis of cattle and sheep. It also causes fowl cholera (Bruner and Gilliespie, 1973; Carter, 1975 and Lennette et al., 1980). The human most commonly becomes infected following an animal bite. However, infections unrelated to this type of trauma have been reported. These include meningitis, empyema and bronchioectasis (Hubbert and Rosen, 1970).

Pasteurella multocida is a small, non-motile, Gram-negative rod which exhibits a bipolar appearance when stained by Gram's, Wright's or Giemsa methods. It is non-sporeforming and freshly isolated strains may be encapsulated. Pasteurella multocida grows well on blood or chocolate agar but it does not grow on MacConkey or Eosin Methylene Blue agar. The differential biochemical characteristics of this microorganism are listed in Table 1 (Cowan, 1974, Lennette et al., 1980, Bruner and Gillespie, 1973 and Graevenitz, 1977).

Apparently, Perroncito (1878) was the first to isolate and describe the microorganism known as the bacillus of fowl cholera.

TABLE 1. BIOCHEMICAL CHARACTERISTICS OF *P. MULTOCIDA*

Characteristic	Sign
Hemolysis	-*
Gas from glucose	-
Acid from:	
Glucose	+++
Mannitol	+
Sucrose	+
Lactose	-
Maltose	-
Raffinose	-
Salicine	-
Xylose	d
Arabinose	d
Sorbitol	+
Urease	-
Simmon's citrate	-
Nitrate reduction	+
Gas from nitrate	-
Indole	+
Gelatin hydrolysis	-
Triple sugar iron (TSI) slant, acid	+
TSI butt, acid	+
Oxidase - cytochrome	+
Esculin hydrolysis	-
Hydrogen sulphide	-
Lysine decarboxylase	-
Arginine dihydrolase	-
Ornithine decarboxylase	+
Catalase	+

d = 16-84% strains positive

-* = Negative reaction

+++ = Positive reaction

In 1880, Pasteur extended the description of the disease and the associated microorganism. Subsequent workers cultured closely related microorganisms from cattle, swine, sheep and other animals suffering from a disease referred to as hemorrhagic septicemia. These strains were so similar in morphology and cultural characteristics that these features could not be used for differentiation. In 1885, Kitt proposed that they be grouped under the name Bacterium bipolare multocidum. Hueppe, in 1886, suggested the name Bacterium septicemia hemmorrhagica for the organism and septicemia hemorrhagica for the disease syndrome. Flugge (1886) separated the microorganisms according to the hosts from which they were recovered by utilizing species names such as bovisseptica, suisseptica, leipseptica, etc. In 1887, Trevisan grouped them in the genus Pasteurella in honor of Pasteur. Lignieres (1901) gave the name pasteurelloses to the group of diseases caused by these microorganisms. In 1939, Rosenbusch and Merchant proposed that the hemorrhagic septicemia microorganisms of various animals be recognized as a single species called Pasteurella multocida. This latter name is now universally employed except in Great Britain where the name Pasteurella septica is commonly used (Merchant and Parker, 1967; Bruner and Gillespie, 1973; and Wilson and Miles, 1975).

Prior to the proposal of the name P. multocida by Rosenbusch and Merchant in 1939, workers recognized that classification based on the animal species from which the microorganism was isolated was not appropriate. Strains recovered from one species of animal would not only infect the homologous species but also heterologous species. Because of this, Roderick in 1922 and Tanaka in 1926 attempted to establish a classification

system based on agglutinating characteristics of the microorganism. In 1929, Cornelius conducted agglutinin-absorption tests to group 17 of 26 P. multocida strains. He demonstrated that antigenic diversity existed among the various strains of P. multocida and that infection by a certain serologic type was not confined to a particular species of animal. Cornelius categorized the different strains into four groups designated as I, II, III and IV.

Yusef, (1935) applied the precipitin-absorption technique to re-examine strains previously studied by Cornelius and confirmed three of the four Cornelius groups. Rosenbusch and Merchant (1939) also demonstrated three types (I, II and III) of P. multocida by agglutinin tests and observed some correlation between serologic behavior and fermentation of xylose, arabinose and dulcitol.

In 1943, Little and Lyon confirmed the existence of three distinct serologic types of P. multocida designated 1, 2 and 3 by passive immunization of mice and rapid slide agglutination tests with specific antisera. Type 1 strains were of avian, bovine, equine and porcine origin; type 2 of bison, bovine, avian and lapine origin while type 3 strains were of avian, bovine, canine, ovine, porcine and murine origin. They observed that monovalent type 1, 2 or 3 antisera protected mice from infection by strains belonging to the homologous type but failed to protect against heterologous types. They indicated that antisera from horses immunized with type 1, 2 and 3 strains consistently protected mice against all virulent P. multocida they studied. Little and Lyon suggested the use of trivalent bacterins and antisera for protection against P. multocida infections. They concluded that type specificity,

virulence and host origin of strains of P. multocida were unrelated.

Roberts (1947) conducted cross protection experiments in mice which indicated that immunologic types existed within the species of P. multocida. He used 21 strain specific antisera to classify 37 strains into four types which were designated I, II, III and IV. Roberts observed that his classification had a loose host specific relationship. Strains from birds were primarily type II while those from the bovine and porcine were generally type I. He also indicated that a moderate correlation existed between immunologic grouping and fermentation reactions. Type I and III strains were arabinose negative and xylose positive while type II strains were arabinose positive and xylose negative. Type IV strains fermented arabinose and dulcitol. Robert reported that type I antisera protected mice only against infection by the homologous type. Type II, III and IV strains conferred full protection against infection with strains of their homologous type and also conferred slight cross protection.

While some workers were classifying P. multocida serologically, others studied dissociation of the organism. Webster and Burn (1926) used an indirect method of illumination to describe three principal colony variants of P. multocida. The colony types were mucoid, smooth and rough. The mucoid colonies were encapsulated and only mildly virulent for rabbits. Smooth colonies were encapsulated, fluorescent and highly virulent while the rough colonies were blue, avirulent and noncapsulated. In 1931, Hughes described an additional colony type which he referred to as intermediate. This type varied in behavior between the smooth and rough strains. Blue and mucoid (M) variants were

frequently recovered from chronic processes and carrier states in chickens and rabbits while smooth and intermediate forms were usually isolated from fowl cholera during the epidemic phase.

Elberg and Ho (1950) studied the colonial dissociation pattern of several strains of P. multocida. They observed that the M variants possessed filamentous cells and were derived from smooth (S) variants. Elberg and Ho also indicated no correlation existed between the morphologic or cultural characteristics and virulence but there was a relationship between the type of fluorescence and virulence of strains.

In 1953, Carter and Bigland examined colony dissociation and virulence of 28 strains of P. multocida isolated from a variety of lesions in several species of animals. They suggested that dissociation in P. multocida proceeded either from smooth to rough or from smooth to mucoid to rough. They also observed that smooth and rough variants were most and least virulent for mice respectively, while the mucoid strains were intermediate.

Hoffenreich (1928) demonstrated that the capsule of P. multocida was polysaccharide which resembled that of other pathogenic bacteria in its physical, chemical, and serologic properties. Based on this, Carter (1951) conducted precipitation tests using a type specific capsular antigen extracted from smooth variants to identify three distinct serologic types designated A, B and C. He confirmed the type specificity of the capsular antigen by a capsular swelling technique.

In 1952, Carter and Annau demonstrated the presence of hyaluronic acid in capsular material of smooth to mucoid variants of type B strains which were not typable with antisera prepared from smooth type B strains.

This polysaccharide, hyaluronic acid complex was serologically inactive and its presence around the cells was considered to be responsible for the untypable nature of the mucoid strains.

Carter (1953) used the precipitation test to study the predominant antigenic types of P. multocida in domestic animals in Canada. Types A, B, and C were all commonly recovered from cattle, swine, bison, and chickens. Mucoid strains were most commonly recovered from chronic processes in swine and chickens. He suggested that commercial bacterins and antisera for the prevention of pasteurellosis should be multivalent. Correlation between fermentation of xylose, arabinose, and dulcitol and serologic type was observed. Type A strains fermented arabinose and dulcitol but not xylose while type B and C strains attacked xylose but not arabinose and dulcitol. At this time, an additional type, D, was demonstrated by the precipitation and capsular swelling tests.

In 1955, Carter introduced the more sensitive indirect hemagglutination test (IHA) to identify the serologic types. Capsular substance was extracted with saline and adsorbed to human type O red cells. Specific antisera were then added to the treated cells and incubated at room temperature. A positive reaction resulted in marked agglutination. Using this method, he typed 124 strains. By comparing his types with those of Roberts, Carter observed that types I, II, III and IV of Roberts were identical with types B, A, C and D of Carter respectively. Carter (1957) used the hemagglutination test to type P. multocida isolated from cattle and swine from different parts of the world. He observed that type A strains were most frequently recovered from cattle with pneumonia while type B were associated with epizootic hemorrhagic

septicemia from animals in the tropics and sub-tropics. Type A and D strains were predominantly recovered from swine with pneumonia. A new strain responsible for hemorrhagic septicemia in cattle in Central Africa was discovered by Carter in 1961 and designated type E.

To eliminate variations due to aging of cells and use of different batches, the IHA was modified to utilize formalized human group O erythrocytes (Carter and Rappay, 1962). In 1963, the type C category was dropped because subsequent work indicated that strains previously classified as such were not an actual capsular type in contrast to the other types (Carter, 1963). As had been demonstrated for mucoid variants of type B strains, the presence of hyaluronic acid in capsular material of IHA untypable type A strains was demonstrated (Carter, 1957). To facilitate typing of mucoid strains, Carter (1972) introduced the method of treating mucoid cultures with testicular hyaluronidase which hydrolysed capsular material and released specific antigen.

The IHA test had proven to be a useful method for typing strains of P. multocida but was somewhat involved and not routinely conducted in diagnostic laboratories (Rimber, 1978 and Carter and Chengappa, 1981). In an attempt to provide a less complicated test, Rimber (1978), introduced the slide coagglutination test for types B and E. Cowan 1 strain Staphylococcus aureus coated with either type B or E absorbed antibody agglutinated in the presence of heat stable, soluble group antigen extracted from cells or present in plasma or liver extracts of infected mice. In another attempt to simplify typing, Carter and Chengappa (1981), introduced counter immunoelectrophoresis as a method for identification of type B and E strains. Capsular substance was extracted with saline

as for IHA and placed in the cathodal well and an equal quantity of antiserum was placed in the anodal well. The agar coated slide was electrophoresed for 30 minutes at 150V and examined for lines of precipitation. They claimed that no cross reactions were observed with heterologous sera even when capsular extracts and antisera were electrophoresed for 30 minutes or more although electrophoresis for longer than 30 minutes reduced specificity. Serum from type B and E infected rabbits also reacted specifically in this test and they suggested that tissue extracts might be used as a source of antigen if sera were not available.

The hemagglutination, capsular swelling and precipitation tests depended upon the presence in cultures of predominance of capsulated organisms. Noncapsulated organisms could not be classified by these methods. So in 1957, Carter introduced the acriflavin test for the differentiation of capsulated from non-capsulated colonial variants. Colonial morphology was studied by obliquely transmitted light and antigenic status by the acriflavin test. Cultures were designated as mucoid, smooth or rough on the basis of colonial morphology and as M, S or R on the basis of their reaction in a 1:1,000 solution of acriflavine. Strains designated M and R were inactive antigenically and formed a slimy precipitate or flocculation respectively, in the presence of acriflavine. Strains designated S segregated into three categories in the presence of acriflavin. They remained in suspension, partially flocculated or flocculated. Strains which flocculated were inactive antigenically although they had a smooth colony morphology. Strains which remained in suspension or only partially flocculated were antigenically active. Carter concluded

that the acriflavin test rapidly identified nonimmunogenic and non-typable strains.

In 1973, Carter described a method to distinguish type D from type A, B and E strains. An aqueous solution of acriflavin was added to a concentrated broth culture. Type D strains formed a characteristic heavy flocculent precipitate.

Carter (1976) proposed creation of five biotypes of P. multocida to be referred to as; the "mucoid biotype" associated with fowl cholera and various infections of cattle, sheep and swine; the "hemorrhagic biotype" which causes epizootic hemorrhagic septicemia in cattle and water buffalo; the "porcine biotype" mostly recovered from swine; the "canine biotype" recovered from dogs and dog-bite infections and "feline biotype" recovered from cats and cat-bite infections. He separated these biotypes by the presence or absence of hyaluronidase decapsulation, acriflavin flocculation, mouse pathogenicity and serum protection tests. Carter stated that determination of biotypes would be useful in assessing the probably pathogenic significance of animal and human isolates.

Although Carter's IHA is one of the most sensitive methods used in identification of antigenic types of P. multocida, large amounts of antisera and human O red cells are required for the test which makes it difficult to perform in a small laboratory. In 1961, Namioka and Murata adapted the slide agglutination test used by Little and Lyon (1943) for typing P. multocida cultures. They mixed a freshly isolated colony of the fluorescent type with antiserum on a glass slide and read the reaction within one minute. Namioka and Murata indicated that when this type of culture was used, the results equalled those of the hemagglutination test. They also observed that when dealing with a culture of mucoid form,

it was better to use very young cultures.

Because of the antigenic complexity of P. multocida, many workers tried to find new methods of classifying them into similar types. In 1972, Heddleston et al., described a gel diffusion precipitin test for serotyping P. multocida strains associated with fowl cholera. They used heat stable antigens extracted with formalinized saline and specific antisera to group the organisms into five serotypes namely 1, 2, 3, 4 and 5. This system has been extended and at present, sixteen serotypes have been identified.

On the basis of the presence of specific capsular antigen on P. multocida, this organism was serologically classified into different strains. However, very little was known about the characteristics of the somatic (O) antigen. Namioka and Murata (1961) examined the serologic characteristics of the O antigens with agglutinin-absorption tests. They treated the organisms with one normal hydrochloric acid to expose the somatic antigen, suspended them in saline, and tested them with homologous and heterologous antisera. All strains tested cross reacted in unabsorbed antisera which indicated they possessed a common antigen. Following absorption of antisera with heterologous strains, specific antigens were demonstrated. They suggested the latter should be referred to as group antigens.

Murata et al., (1963) conducted O antigenic analysis on 156 cultures and divided them into ten O groups. The antigenic formula of each serotype was written as an Arabic numeral for the O antigen and as letter for the capsular antigen. When they correlated their O groups with Carter's capsular types, 12 serotypes were described. In 1967,

Namioka described 3 additional serotypes. Types 5:A and 9:A were pathogenic for fowl while 1:a, 3:A and 7:A caused pneumonia in cattle, swine and sheep.

Pasteurella multocida has the ability to infect a broad spectrum of hosts and for this reason, a variety of methods have been employed to classify similar types of this organism and to correlate the type with a specific disease. In 1979, Brogden and Packer conducted an experiment to compare five of the serotyping systems, namely Roberts cross protection test, Little and Lyon's plate agglutination test, Carter's indirect hemagglutination test, Heddleston's gel diffusion precipitin test, and Namioka's plate and tube agglutination test. Reference cultures were examined by the typing system from which they were described and by each of the other typing systems except Roberts. They observed that when reference cultures were examined by the typing system from which they were described, the results agreed with those which were published. However, serotypes examined by one typing system generally did not correlate with serotypes determined by another system. Cultures of a single serotype in one system often represented more than one serotype in another system. Table 2, which is reproduced exactly from Brogden and Packer (1979), demonstrates this incompatibility. They concluded that because of the antigenic complexity of P. multocida and the nature of the antigens involved in each test, there was no reliable correlation between serotypes of the various systems.

Pasteurella multocida possesses the enzyme tryptophanase which catalyzes the cleavage of tryptophan to indole, pyruvic acid and ammonia. The parent compound of indole and other natural indoles, such as three-methylindole, is the amino acid L-tryptophan (Gunsalus et al., 1955).

TABLE 2--Serotyping Results of *P. multocida* Type Cultures

Type	Little and Lyon agglutination test	Carter capsular- typing methods	Namiooka capsular- somatic test	Heddleston gel diffu- sion precipitin test
HEDDLESTON				
1	1	A	3A	1
2	-	B	7A	2,5
3	1	A	8A	3
4	-	A	3,8A	4
5	-	A	4D	5,2
6	1	A	9A	6
7	1	A,SLB,E	7,9-	7
8	1	A	3A	8
9	-	A	5,7A	9
10	-	-	1,7A	10
11	1,3	D	2,4D	11
12	-	A	1A	12
13	1	A,SLB,E	-	13
14	1	A	5A	14
15	1	D	1,2D	15
16	-	A	9A	16
CARTER				
A	-	A	1,9A	3
B	2	B	6B	2,5
D	-	D	1,3D	11
E	-	E	6E	2,5

TABLE 2 (continued)

Type	Little and Lyon agglutination test	Carter capsular- typing methods	Namioka capsular- somatic test	Heddleston gel diffu- sion precipitin test
NAMIOKA				
1A	-	A	1,7,9A	4
2D	-	D	2,4D	3
3A	-	A	3A,D,7A	10,11
4D	-	D	4D,8A	3
5A	-	A	5A	1
6B	1,2	B	6B	2,5
7A	1,3	A	3,7A,3D	10
8A	-	A	8A,4D	3
9A	1	A	9A	3
ROBERTS				
I	1,2	B,E	6B,E	2,5
II	-	A	5A	14
III	1	A	2,4D	3
IV	-	-	1,2-	1
V (P-2251)	-	D	-D	3
V (P-2254)	-	A	-A	3
V (P-2256)	-	D	-D	3,s112
LITTLE AND LYON				
1	1	D	2,4D	3
2	2	-	7A,4D	2,5
3	3	-	7A	10,11

Jepson, 1969, Long, 1961 and Knoeman et al., 1979). Indole is a benzyl pyrrole consisting of a benzene ring attached to the pyrrole ring (Figure 1). It is a neutral compound having a white to yellowish color, an unpleasant odor in high concentrations with a pleasant odor in low concentrations, and is soluble in alcohol, ether, hot water, and fixed oils but insoluble in mineral oil and glycerol. This volatile compound has a melting point of 52°C and boiling point of 254°C (Hawley, 1971).

Three-methylindole (3 MI, skatole) is a methylated indole (Figure 1) with a methyl group attached to the 3 carbon atom on the pyrrole ring. It is similar in physical and chemical properties to indole. Three-methylindole is a white crystalline substance, browning upon aging and is soluble in hot water, alcohol and benzene. The melting point of this compound is between 93°C to 95°C and the boiling point is 265°C . It has a very unpleasant odor which is partly responsible for the characteristic odor of feces (Hawley, 1971).

Indole, 3 MI and other indolic compounds such as indoleacetic acid (IAA) and 5-methylindole (5 MI) can be detected with several different reagents (Blazeric and Ederer, 1975). Ehrlich's reagent, which is composed of p-dimethylaminobenzaldehyde, absolute ethyl alcohol and concentrated hydrochloric acid, is the most sensitive and useful (Knoeman et al., 1979). It reacts with indole compounds to form a red color. The chemical reaction is based on the formation of a rosindole dye when two indole molecules react with the aldehyde group of one molecule of p-dimethylaminobenzaldehyde (Blazeric and Ederer, 1975 and Knoeman et al., 1979). Ehrlich's reagent reacts with a number of other indolic and related compounds to form colors which may be pink, orange, purple, blue, yellow, gray or brown. Therefore, it is not a specific test for

FIGURE 1

Structural formula of L-tryptophan, indole-acetic acid, indole and 3-methylindole.

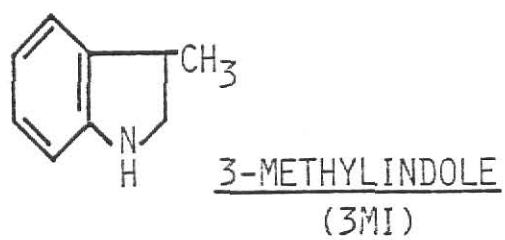
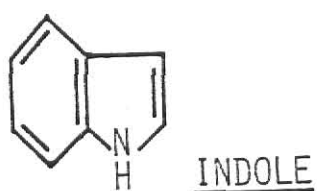
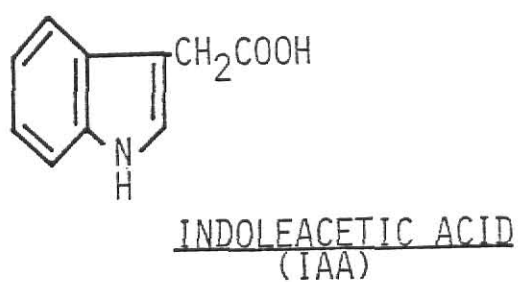
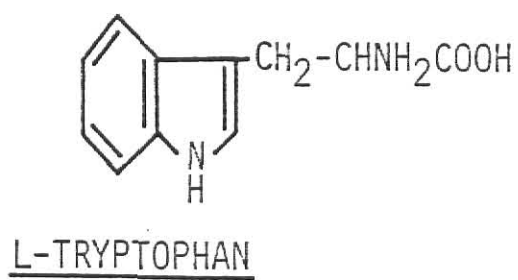


FIGURE 1.

the presence of indole (Jepson, 1969). Other reagents which have been utilized to detect indole are Kovac's, oxalic acid, and hydrooxalamine hydrochloride.

The major route of tryptophan degradation in man and animals is by the kynurenine pathway (Nakoneczna et al., 1969) (Figure 2). In this pathway, the indole ring of tryptophan is cleaved by tryptophan pyrrolase (2,3-dioxygenase) to form N-formylkynurenine which is further degraded to kynurenine. Kynurenine is then converted to 3-hydroxykynurenine which in turn is converted to 3-hydroxyanthranilic acid and finally to nicotinic acid which is an important vitamin in the B-complex group.

Bacteria which are normally present in the intestinal tract of man and animals, degrade tryptophan by the indole pathway (Figure 2) (Nakoneczna et al., 1969). In this pathway, L-tryptophan is converted to IAA which undergoes decarboxylation to 3 MI and indole (Yokayama et al., 1974, Carlson et al., 1972, Hammond et al., 1979, and Hammond and Carlson, 1980). Indole and 3 MI are then voided in feces, absorbed from the intestinal tract and excreted in urine or metabolized by certain organic systems. Some indole absorbed by the blood is metabolized in the liver to indoxyl which combines with potassium sulphate to form indican. Indican is then excreted in urine (Forbes et al., 1935 and Nakoneczna et al., 1969).

Three-methylindole is metabolized by the mixed-function oxidase enzyme system (MFO). These enzymes, also called monooxygenases, are located in the smooth endoplasmic reticulum (SER) of cells of the lungs and liver. They participate in catabolism and detoxification of drugs and toxic substances. The MFO hydroxylates substances by introduction of one oxygen atom. Hydroxylated substances are then water soluble and

FIGURE 2

Diagram of tryptophan metabolism via the kynurenine and indole pathways.

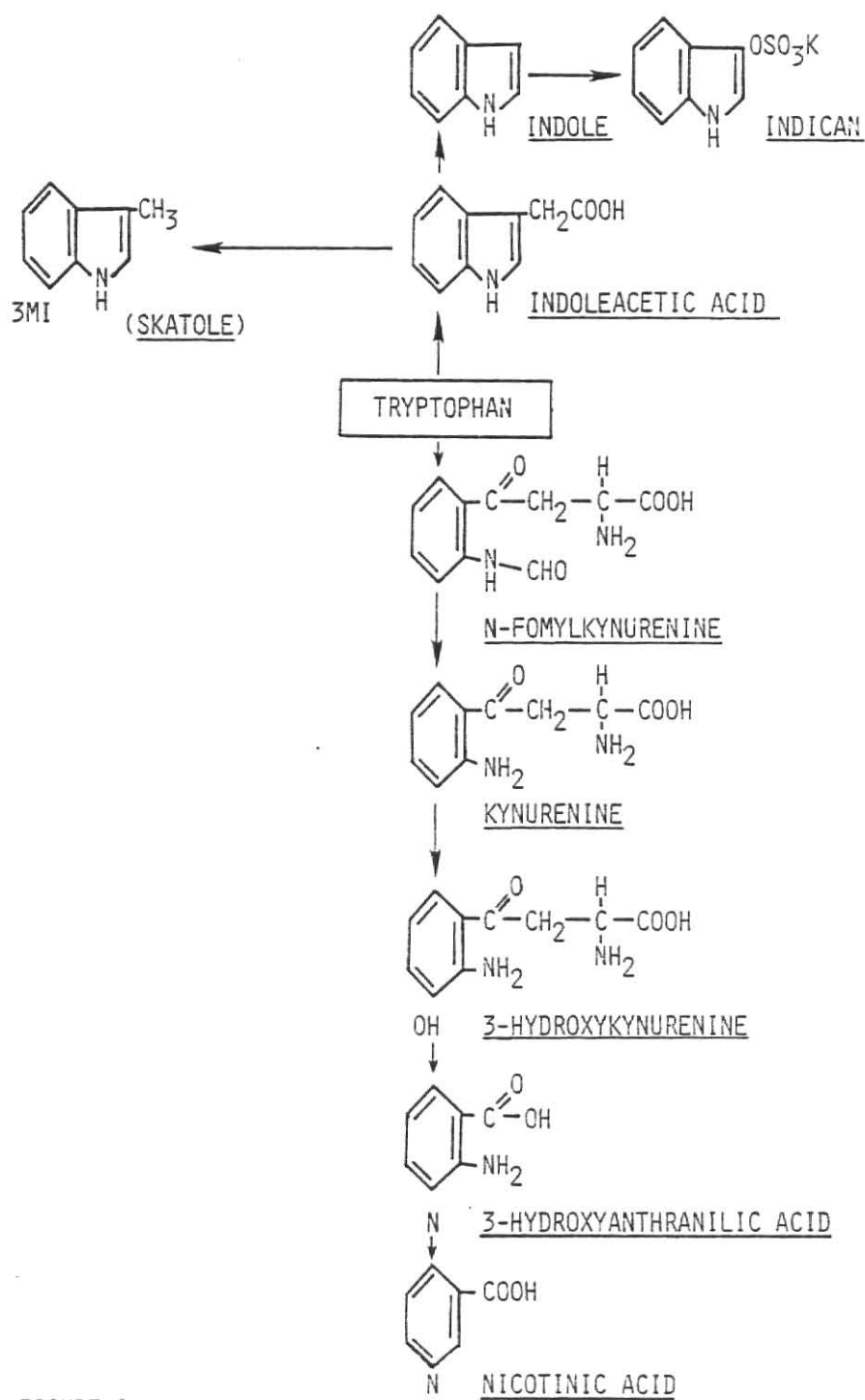


FIGURE 2.

excreted in the urine (Lehninger, 1975 and Mahler and Cordes, 1971). Certain of these metabolites may be toxic. Bray and Carlson (1977) suggested two pathways of 3 MI metabolism by the MFO system. A major pathway in which 3-methyloxindole was produced and a minor pathway associated with formation of indole-3-carboxylic acid.

Indole and 3 MI have been associated with the etiology of several diseases and their relationship to several others is being investigated. One of these diseases is acute bovine pulmonary emphysema (ABPA). This disease has been referred to as bovine asthma, summer snuffles, hay fever, pulmonary emphysema, pulmonary edema and fog fever. The condition usually occurs after cattle are changed from short, dry, grass to lush, green pasture (Person, 1954, Baldwin, 1954, Tucker, 1962, Moore, 1952, Tucker and Maki, 1962 and Carlson et al., 1968). It is characterized by a sudden onset with severe expiratory dyspnea (Moore, 1952. Baldwin, 1954, and Tucker and Maki, 1962).

The first cases of ABPA in North America were reported by Schofield (1927). In the United States, the disease is a major cause of economic loss in the western states of Utah, Wyoming, California and Texas (Heron and Suther, 1979). Although this disease is common, its etiology remained unknown for a long time (Maki, 1963). Initially, it was thought to be a combination of climate and allergy to legumes or other plants (Moore, 1952, Fuechsel, 1952 and Bealth, 1954). Dictyocaulus sp and toxins of Clostridium perfringens were considered by some workers to be important factors in the etiology of the disease (Solman, 1952, Michel, 1954 and Schofield, 1948). Results of investigations by other workers did not support this hypothesis (Maki and Tucker, 1962, Breeze et al., 1974 and

Soliman et al., 1977).

An acute respiratory syndrome resembling ABPA was observed in cattle when Dickinson et al., (1967) administered DL-tryptophan (DL-T) orally to cattle during an experiment to study enzymes associated with experimental bloat. Some of the animals died in acute respiratory distress and post-mortem examination revealed lesions similar to those observed in cases of ABPA. When the experiment was repeated, the same results were obtained.

Carlson et al., (1968) administered DL-T to groups of cattle by oral, intraperitoneal and intravenous routes. Pulmonary emphysema was produced only in those animals which received the amino acid orally. However, plasma tryptophan levels in each group were similar. They concluded that a product of ruminal tryptophan metabolism, not the amino acid itself, was possibly the causative factor of this syndrome. In the same study, sheep which were dosed with DL-T at the same level, failed to develop respiratory disease. This indicated a difference in metabolism of tryptophan between sheep and cattle. Later Dickinson (1970) demonstrated that goats also did not develop pulmonary disease when administered tryptophan by the oral route. In 1972, Carlson et al. determined that only the L-isomer of tryptophan produced the syndrome.

In the same experiments, they anaerobically incubated L-tryptophan (LT), D-tryptophan (DT) and indole acetic acid (IAA) with stained rumen fluid. The fermentation metabolites were separated by ion-exchange chromatography and identified by thin layer chromatography (TLC). They observed that the chief product of LT degradation was 3 MI. Indole and IAA were also present in smaller quantities. The D isomer of

tryptophan was not converted to any of these compounds. However, IAA was converted to 3 MI by ruminal microorganisms. They then tested the ability of 3 MI to induce ABPA by administering the compound orally and intravenously to cattle. The disease was induced by both methods of administration. It was concluded that 3 MI was the ruminal metabolite of tryptophan responsible for the development of ABPA. Subsequent experiments confirmed these observations and also demonstrated that this compound induced this disease in sheep and goats (Carlson et al., 1975, Yokoyama et al., 1975, Terry et al., 1976 and Bradley et al., 1978).

Bradley and Carlson (1974) devised a gas liquid chromatographic method (GLC) to determine indole and 3 MI concentrations in bovine plasma. They extracted plasma with methylene chloride, evaporated the extract under reduced pressure and concentrated it under nitrogen. They used 21% Carbowax 4000 on Chromasorb W-DMCS (60/80 mesh) as a stationary phase. With this technique, a detection limit of 0.5 ng was reported. Bradley and Carlson confirmed the presence of indole and 3 MI in extracts by TLC utilizing chloroform: cyclohexane: diethylamine (5:4:1 U/V) as the solvent and Ehrlich's reagent as the detection agent.

In 1974, Yokoyama and Carlson used GLC and TLC to identify metabolites of tryptophan and selected other related indole compounds incubated with ruminal microorganisms in vitro. They confirmed that 3 MI was the major metabolite of LT degradation. They also observed that both indole and 3 MI were produced when indolepyruvate, indolelactate and indolealdehyde were incubated under these conditions and that indole acetaldehyde was converted to IAA and tryptophol. Under the same conditions, 38% of the radioactivity of Carbon 14 labelled IAA was incorporated into 3 MI.

Normal rumen fluid also contained indole and 3 MI but in small quantities. Yokoyama and Carlson demonstrated that glucose reduced the formation of both indole and 3 MI from LT resulting in the accumulation of IAA. Antibiotics, especially the aminoglycosides kanamycin and neomycin, reduced conversion of LT to indole and 3 MI. It was suggested that the major pathway of 3 MI formation from LT was by decarboxylation of IAA and that more than one bacterial species was involved in the production of this compound by this process (Figure 3).

With the establishment that microorganisms converted LT to indole, 3 MI and IAA many workers tried to isolate and identify the species of bacteria which were responsible for tryptophan degradation. In 1975, Chung et al., investigated intestinal anaerobes to determine which produced IAA. They grew 23 strains in liquid media and screened the samples for the presence of IAA by TLC. Of the 23 strains studied, only Bacteroides fragilis subsp thetaiotaomicron, Escherichia coli, and Citrobacter sp. produced IAA. Yokoyama et al., (1977) isolated a bacterium capable of decarboxylating IAA to 3 MI. The isolated 3 MI-producing bacterium was determined to be a Lactobacillus sp. by its physiologic, metabolic and biochemical characters. They also observed that the isolated strain did not form 3 MI directly from tryptophan.

Although it was established that 3 MI was responsible for the production of ABPA, some investigators believed that toxic metabolites of this compound by the MFO system and not the compound itself were involved in the mechanism of the disease. In 1979, Bray and Carlson studied the relationship between pulmonary toxicity of 3 MI and the MFO system. They administered 3 MI to three groups of goats by intravenous

FIGURE 3

Pathway for the fermentation of L-tryptophan and related indolic compounds by ruminal microorganisms in vitro.

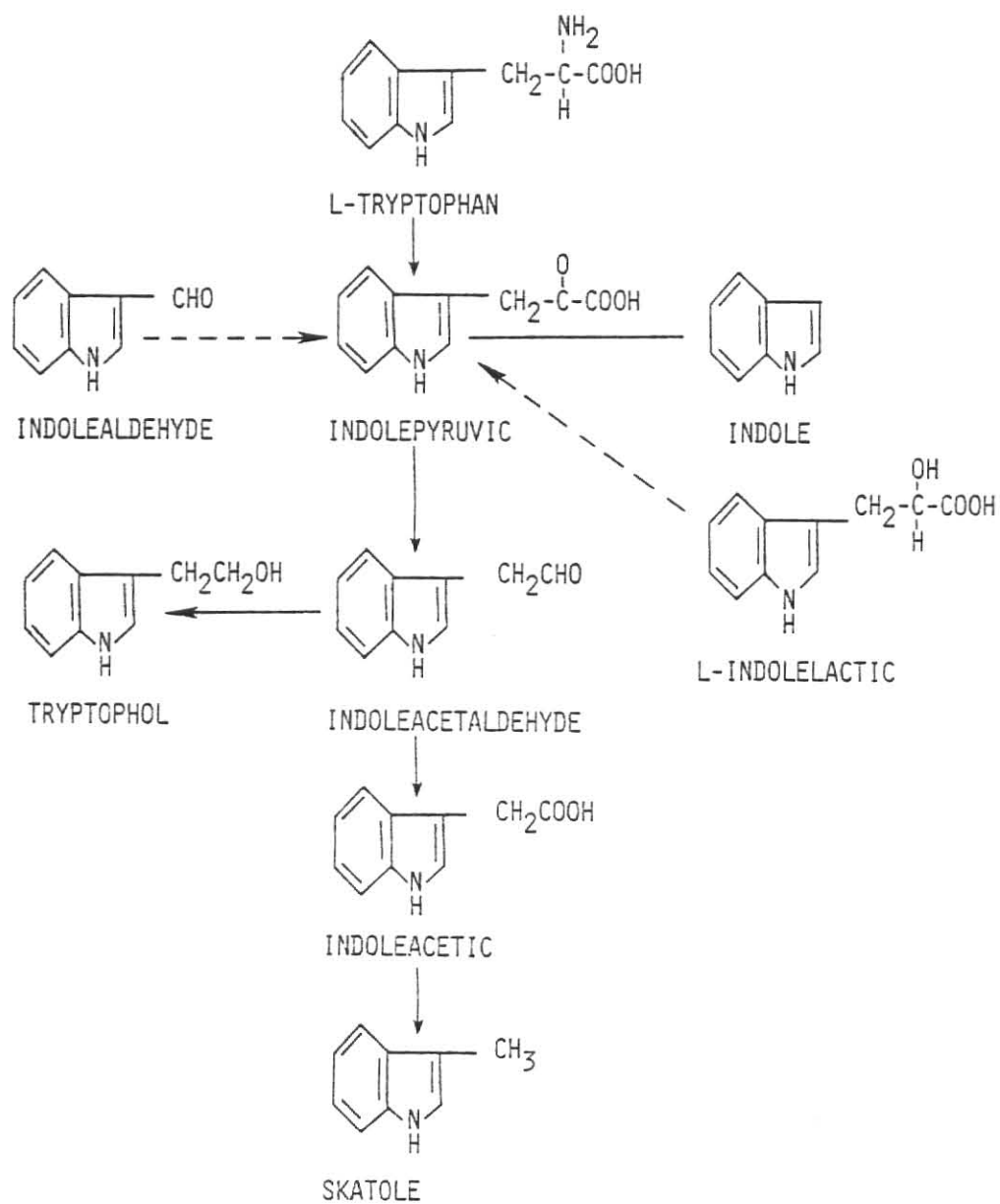


FIGURE 3.

infusion. One group had received a phenobarbital (PB) pretreatment, which increases MFO activity, the second group had received a piperonyl butoxide (BT) pretreatment, which decreased MFO activity, and the final group received no pretreatment. All goats except those pretreated with BT developed ABPA. Pulmonary lesions in goats pretreated with PB were more severe than the controls. The clearance of 3 MI from plasma of goats pretreated with PB was shorter than that in controls or those treated with BT. Altered plasma clearance rates indicated that increased MFO activity increased the rate of 3 MI degradation and suggested that the MFO system was directly involved in metabolism of 3 MI. This conclusion was supported by the observation of an increase in the amount of SER in the most severely affected pulmonary cells. They concluded that 3 MI was not toxic in itself, but was metabolized by the MFO system into other pneumotoxic substances.

Indole and 3 MI also possess biological activity which enables them to cause disease directly. A characteristic is lipophilicity which enables them to interact with lipid molecules present in cell membranes causing disruption (Nakoneczna et al., 1969). Rogers (1969) demonstrated that indole, 3 MI, and certain other aromatic compounds produced hemolysis of rabbit erythrocytes. The extent of hemolysis was proportional to lipophilicity. Prior to Roger's experiments, Forbes and Neale (1935) had discovered that the concentration of indole in urine of human patients with chronic arthritis was higher than in normal urine. Subsequently, they produced chronic arthritis in joints of rabbits by intracapsular injections of indole, 3 MI, and indolepropionic acid. They suggested that products of tryptophan putrefaction were causative agents in

production of rheumatoid arthritis.

In 1969, Nakoneczna et al. injected metabolites from the kynurenine and indole pathways intraarticularly into rabbits to determine their arthrogenicity. Both indole and 3 MI which are highly lipophilic induced a rheumatoid-like arthritis, while kynurenine, 3-hydroxykynurenine 3-hydroxyanthranitic acid as well as indican, oxindole and isatin, which were not so lipophilic did not.

Although indole and 3 MI are normally produced in the gastrointestinal tract of man and animals, any condition which may cause excessive production of these compounds or may allow 3 MI to bypass hepatic detoxification, as in liver disease, pose potential danger to lungs (Huang et al., 1977).

MATERIALS AND METHODS

Preparation of Inocula

Rabbits

Pasteurella multocida (#1062*, type A) was obtained from the stock culture collection of the Department of Laboratory Medicine, Kansas State University. The organism was inoculated on a tryptic soy agar** plate enriched with five percent citrated bovine blood and incubated at 37C overnight. A loop full of the overnight growth was used to inoculate 10 ml of sterile brain heart infusion broth (BHI)*** in a 25 ml screw capped Erlenmeyer flask and incubated at 37C for five hours. The percentage transmission of the culture was adjusted to 84 percent with a Coleman spectrophotometer**** at wavelength of 540 nm, by diluting the culture with sterile BHI. Ten fold dilutions of the culture were made in sterile normal saline, then 0.1 ml of each dilution was spread on five blood agar plates. The plates were incubated at 37C for 24 hrs and the colonies counted. The culture contained 9.9×10^8 colony forming units (cfu) per ml

Pasteurella hemolytica (type 1, strain #129*****) was also obtained from the stock culture collection of the Department of Laboratory Medicine and was treated as previously described for P. multocida except for the

*Dr. G. R. Carter, College of Veterinary Medicine, Michigan State University, East Lansing, Michigan.

**Gibco Diagnostic, Madison, Wisconsin.

***Difco Laboratories, Detroit, Michigan.

****Coleman, Maywood, Illinois.

*****Dr. G. Frank, National Animal Diseases Centre, Ames, Iowa.

following differences. The BHI broth was incubated at 37C for eight hours. The transmission was checked and adjusted to 81 percent as described before and plate counts conducted were found to contain 1.1×10^9 cfu per ml.

Mice

One colony of an overnight growth of P. multocida #1062 from a blood agar plate was used to inoculate 50 ml of sterile BHI broth in 125 ml screw capped Erlenmeyer flask and incubated at 37C for 24 hrs. Likewise, a colony of an overnight growth of P. hemolytica #129 from a blood agar plate was transferred into 50 ml of sterile BHI broth and incubated at 37C for 24 hours.

Inoculation of Animals

Rabbits

Rabbits used in this study were Pasteurella-free New Zealand white,* weighing approximately five pounds each. Rabbits 1, 2 and 3 were inoculated conjunctivally using sterile tuberculin syringes**. Each rabbit received 1×10^7 cfu of P. multocida suspended in 0.1 ml sterile saline (Al-Lebban, 1981). This concentration had been previously demonstrated to be the minimal lethal dose. Rabbits 4 and 5 were inoculated in the same way with 1×10^7 cfu of P. hemolytica suspended in 0.1 ml sterile saline. This group served as controls inoculated with a microorganism which does not produce indole or 3 MI when incubated in the presence of tryptophan. Rabbits 6 and 7 served as controls and were inoculated as described before with 0.1 ml sterile BHI broth.

*Buckshire Corporation, Parkview, Pennsylvania.

**Becton, Dickinson & Company, Rutherford, New Jersey.

Mice

Forty mice were each inoculated with 0.1 ml of a P. multocida 24 hrs broth culture intraperitoneally. Thirty mice which served as a control group of animals infected with an indole negative microorganism were each inoculated by the same route with 0.2 ml of a 24 hrs P. hemolytica broth culture. Thirty additional mice served as uninoculated controls.

Collection of Specimens

Blood samples

Three ml of blood was collected from the ear vein of each rabbit using 3 ml monoject sterile disposable syringe* rinsed with heparin solution 1000 USP units/ml**. Samples were collected three hours perinoculation, just before inoculation, and at 3, 6, 9, 12, 15, 18, 24, 30 and 36 hours post inoculation. Each sample was put in a polypropylene tube*** and centrifuged at 3000 rpm for 10 minutes on a General Laboratory Centrifuge (GLC-2)****. The plasma was removed with Pasteur pipetes, put in polypropylene tubes, labelled, and stored in the freezer at -20C.

Organs

Control and P. hemolytica inoculated rabbits were euthanitized 36

* Sherwood, Deland, Florida.

** Lypho-Med, Inc., Chicago, Illinois.

*** Falcon, Oxnard, California.

**** Van Sorvall, Inc., Newtown, Connecticut.

***** Dynatech Laboratories, Inc., Alexandria, Virginia.

hours post inoculation. Pasteurella multocida infected rabbits died within 24 hours post inoculation. All rabbits were necropsied and livers, kidneys, hearts, spleens and lungs collected. Each organ was put in a preweighed Stomacher Lab Blender plastic bag****, weighed and stored frozen at -20C.

Normal mice and surviving P. hemolytica infected mice were euthanitized with chloroform. Organs of these mice and of those that died due to P. multocida infection were harvested as described for rabbits. Similar organs in each group of mice were pooled in Stomacher Lab Blender plastic bags, weighed, and stored in the freezer at -20C.

Broth Culture

Pasteurella multocida and P. hemolytica were grown in 20 ml BHI. Twenty ml of uninoculated BHI broth served as control.

Extraction of Indolic Compounds

Organs

About 2 volumes of 0.1 M phosphate buffered saline (PBS) pH 7.2 was added to each plastic bag and the contents minced with the Stomacher Lab Blender 80*. The minced tissue was refrozen until utilized. To extract the indolic compounds, the minced tissue was thawed at room temperature, placed in a screw capped Erlenmeyer flask, and 10 volumes of methylene chloride added. A teflon coated magnetic stirring bar was placed in the flask and the mixture stirred on a magnetic stirrer**

*Dynatech Laboratory, Inc., Alexandria, Virginia.

**Corning, Corning, New York.

***Whatman, Inc., Paper Division, Clifton, New Jersey.

for 10 minutes. The emulsion was then poured into a 100 ml separatory funnel and allowed to dissociate. The lower methylene chloride phase was filtered through Whatman phase separating paper*** into a clean screw capped Erlenmeyer flask. Those emulsions which would not separate spontaneously were placed into 40 ml conical graduated screw capped glass tubes and centrifuged at 2000 rpm for 10 minutes in an International Centrifuge*. The methylene chloride layer was collected with a 5 ml pipette and filtered as described before. Each tissue was extracted three times and pooled in a round bottom flask with a TS 24/40 female joint and evaporated under reduced pressure on a rotary evaporator** to approximately 7 ml. The temperature of the water bath was kept at 28C. The extract was quantitatively transferred with a Pasteur pipette into a 20 ml glass stoppered test tube, the flask rinsed with four 1 ml washes of methylene chloride and the washes added to the extract. In an attempt to remove interfering compounds, the extracts were treated with a selective adsorbent with a magnesium-silica gel catalyst (Florisil). Two grams of Florisil*** were added to the extract and shaken for 1 hour on a wrist action shaker**** and centrifuged at 2000 rpm for 10 minutes to separate the extract from the Florisil. The extract was transferred to a 40 ml conical graduated glass tube with a Pasteur pipette. The

* International Equipment Company, Boston, Massachusetts.

** Rinco Instrument Co., Inc., Greenville, Illinois.

*** Fisher Scientific Company, Fairlawn, New Jersey.

**** Burrell Corporation, Pittsburg, Pennsylvania.

***** Dade Diagnostic, Inc., Miami, Florida.

sedimented Florisil was re-extracted two times with 10 ml of methylene chloride. Following each extraction the Florisil was sedimented by centrifugation and the supernatant pooled. The extract was evaporated under nitrogen to 0.1 ml per gram of tissue. Twenty microliters of the concentrated extract was distributed into 200 μ l accupette pipettes which had been sealed on one end with a flame to form a vial. The other end of the vial was then heat sealed. Extracts were stored at -20C until utilized.

Plasma

Plasma samples were extracted and stored as described for tissues but were not adsorbed with Florisil.

Cultures

Broth cultures of P. multocida and P. hemolytica as well as uninoculated BHI broth were extracted, adsorbed and stored as described for tissues.

EXAMINATION OF EXTRACTS FOR INDOLIC COMPOUNDS

Tube Test with Ehrlich's Reagent

Approximately 60 μ l of each extract was placed in a 6 x 50 mm glass test tube and overlaid with approximately an equal volume of Ehrlich's reagent (Koneman et al., 1979). Tissue and culture extracts were tested prior to and after treatment with Florisil. A positive test for indolic compounds was indicated by the development of a pink color at the interface (Koneman et al., 1979). To determine the smallest amount of indole and 3 MI which were detected by this procedure, solutions of commercially

obtained certified reagent grade indole* and 3 MI** were prepared separately in methylene chloride in concentrations which contained from 1 ng/ μ l to 0.125 ng/ μ l. These solutions were examined in the same manner as the extracts.

Thin Layer Chromatography (TLC) of Extracts

Extracts were examined for the presence of indole and 3 MI by TLC using 20 x 20 cm silica gel coated glass plates*** which contained a dye that fluoresced under an ultraviolet light (UV) at a wavelength of 254 nm. Eighty μ l of each extract was spotted on the plates. A single plate accommodated 5 spots. Extracts of the same tissue from controls, P. multocida infected, P. hemolytica infected, and 2 standards containing indole and 3 MI were spotted on the same plate. Following spotting, plates were developed once in a chromatography chamber utilizing chloroform: cyclohexane: diethylamine (5:4:1 V/V) as solvent (Heacocke and Mahon, 1963). Plates were then observed under UV light to determine if indole or 3 MI was present in the extract as indicated by quenching of fluorescence. The minimum amount of indole or 3 MI which was detected by this procedure was determined by spotting varying quantities of a standard solution containing 20 ng/ μ l of each compound on a plate. Following development, this plate was observed under UV light to observe the smallest quantity which quenched the fluorescence.

* Fisher Scientific, Fairlawn, New Jersey.

** Eastman Kodak Co., Rochester, New York.

*** E. Merck, Darmstadt, Germany.

**** General Glassblowing, Richmond, California.

The position of various compounds which migrated with the solvent were marked on the plates with a lead pencil and then transferred to tracing paper. Using a chromatography sprayer the plates were then misted with Ehrlich's reagent (Jepson, 1960) to indicate the positions and presence of indolic compounds. Apparent retardation factor (R_f) values of spots which upon development, and stained with Ehrlich's reagent were characteristic of indole and 3 MI, were calculated by dividing the distance they travelled from the origin by the distance the solvent front moved from the origin. The R_f values of known indole and 3 MI standards were compared to values of spots from specimen extracts.

Gas Liquid Chromatography (GLC)

Attempts were made to identify and quantitate indole and 3 MI in extracts by GLC utilizing a Fisher Model 2400 instrument* equipped with hydrogen flame ionization detectors. Silane treated glass columns 6 feet long with an internal diameter of 2 mm were utilized. A variety of column packing materials were used. These included; 3% SP-2250 on 80/100 Supelcoport, 3% SP 2100 on 100/120 Supelcoport, 1% SP 1240 DA on 100/120 Supelcoport, 1% SP 1000 on 100/120 Supelcoport**, 21% Carbowax 4000 on 60/80 chromosorb W-AW-DMCS and 10% Carbowax 4000 on 60/80 chromosorb W-AW-DMCS***. The carrier gas was helium at flow rates from 20-50 ml/minute. Hydrogen and air flow to support the flame were optimized to obtain maximum sensitivity at each different helium flow rate. In attempts to obtain satisfactory results, injector, column oven and detector

* Fisher Scientific, Fairlawn, New Jersey.

** Supelco, Inc., Bellefonte, Pennsylvania.

*** Alltech Associates, Deerfield, Illinois.

temperatures were varied depending upon the particular column packing used and the results obtained. Injector and detector temperatures were at least 50C above that of the column oven. One microliter quantities of extracts, to which indole and 3 MI were added or standard solutions of these compounds in methylene chloride were injected in the on column mode.

Column Chromatography of Tissue and Culture Extracts

First attempts to remove compounds from extracts which would interfere with indole and 3 MI isolation in TLC and GLC were conducted by column chromatography utilizing activated silicic acid (Unisil*) as the packing material. Thirty by 1 cm glass Econocolumns** were slurry packed with the adsorbent suspended in methylene chloride. One half ml of the extract was concentrated to 0.1 ml by evaporation under nitrogen and carefully applied to the top of the packing. Elution by gravity flow was with methylene chloride at a flow rate of 25 drops per minute. Fractions of 96 drops each were collected in separate tubes utilizing a fraction collector*** in the drop count mode. Initial experiments to determine the fractions in which indole and 3 MI were eluted from the column were conducted with extracts to which approximately 300 ng of each of the compounds had been added. The presence of indole and 3 MI in fractions was detected by the Ehrlich's tube test.

*Clarkson Chemical Company, Inc., Williamsport, Pennsylvania.

**Bio Rad Laboratories, Richmond, California.

***Isco, Lincoln, Nebraska.

Fecal culture of rabbits and mice

Fresh fecal material from three rabbits and three fecal specimens from a cage containing approximately 30 mice were cultured to estimate the numbers of Escherchia coli present. These specimens were each streaked for isolation on McConkey agar^{*} plates, incubated at 37C overnight and numbers of E. coli colonies estimated. Colonies which were typical of this species were picked and examined in differential media for identification by standard methods (Koneman et al., 1979).

* Difco Laboratories, Detroit, Michigan.

RESULTS

All rabbits and mice which were infected with P. multocida became ill and died within 24 and 8 hours respectively. The mice infected with P. hemolytica initially sickened and 8 died, but the remainder appeared to begin to recover and were euthanized at 10 hours post inoculation. Rabbits infected with P. hemolytica did not become ill and were euthanized at 36 hours post inoculation.

Attempts to remove interfering compounds from extracts by column chromatography with Unisil were not successful. When extracts to which indole and 3MI were added were chromatographed, fractions 23-50 contained the compounds as indicated by the Ehrlich's tube test. However, neither indole nor 3 MI could be detected in this test in any of the fractions when either tissue or culture extracts were treated in this manner even though they were positive to the Ehrlich's tube test prior to chromatograph. Since this method did not prove satisfactory, extracts were treated with Florisil as described. With this method, extracts which were positive in the Ehrlich's test prior to treatment were also positive after treatment.

Utilizing varying concentrations of standard solutions in the Ehrlich's tube test, the smallest amount of either indole or 3 MI that could be detected was 0.25 ng/ μ l. Since during the extraction process, one gram of tissue was concentrated to 0.1 ml, this test would detect either indole or 3 MI at a level of 25 ng/gram of tissue or 25 parts per billion.

Results of testing tissue and culture extracts by the Ehrlich's method are presented in Table 3. No extracts prepared from rabbit tissue or plasma contained Ehrlich reacting substances at a level which

TABLE 3. Results of the Ehrlich's tube test on rabbits and mice tissues, and culture

Extract	Uninfected	Pasteurella multocida infected	Pasteurella hemolytica infected
<u>Mouse</u>			
Kidney	++	+	+
Liver	+	+	+
Heart	-**	+	+
Lung	-	+	+
Spleen	-	+	+
<u>Rabbit</u>			
Kidney	-	-	-
Liver	-	-	-
Heart	-	-	-
Lung	-	-	-
Spleen	-	-	-
Culture	-	+	-

++ Pink color development at the interface

-** No pink color development at the interface

this test detected. Liver and kidney extracts of tissues from uninfected, P. multocida infected, and P. hemolytica infected mice all contained sufficient Ehrlich reacting substances to be detected by the test. Heart, spleen and lung extracts of both P. multocida and P. hemolytica infected mice but not uninfected mice reacted positively to the test.

The minimal amount of either indole or 3 MI which was detected by TLC was 400 ng. No tissue extracts from either mice or rabbits possessed sufficient levels of Ehrlich's reacting compounds to be detectable by TLC, either at the same Rf values as standards or at other Rf values.

Culture of mouse fecal material on MacConkey agar plates resulted in growth of typical E. coli colonies which were too numerous to count. However, of the three rabbits fecal specimens cultured, only one yielded colonies which were typical of E. coli. Only two typical colonies of this species were observed.

Although variety of different column packing materials and operational methods were attempted, GLC did not provide a satisfactory method for analyzing tissue or culture extracts for the presence or concentration of indole or 3 MI. Utilizing standard solutions of these compounds, the lowest concentration which produced adequate peaks for quantitation or calculation of retention time was 5 ng/ μ l. Tissue and culture extracts prepared in this way produced chromatograms with numerous interfering peaks, some at or near the same retention times of the compound which precluded an accurate analysis. Operating conditions which provided most optimal results with standard solutions utilized 3% SP 2250 on 80/100 Supelcoport as the packing material. Helium, hydrogen, and air flows

were 40, 32 and 300 ml/minute respectively. The temperature of the injection port was 165C and the detector 250C. The column was set at 70C for injection, held at this temperature for 5 minutes, and then increased by program to 170C at a rate of 10C/minute. When the column oven was maintained at 170C during periods of inactivity, no appreciable column bleed was detected with this packing material during subsequent programmed temperature increases. Thus it was not necessary to utilize the dual column differential mode to produce acceptable chromatograms.

DISCUSSION

The results of this study tended to indicate that P. multocida did not produce indole during active infections in either mice or rabbits. This was particularly evident in the rabbit studies since no Ehrlich reacting compound (ERC) were detected in any tissue or plasma extract by the tube test which detected such compounds at a minimum level of 25 ng/gram.

The results obtained in the mouse studies were not as easily interpreted. Ehrlich reacting compounds were found in both liver and kidney extracts of all three groups of mice. It is known that certain bacteria in the intestinal flora produce both indole and 3 MI from tryptophan and these compounds are either eliminated in feces or absorbed to be detoxified in the liver and excreted by the kidney (Nakoneczna, et al., 1969). Thus it was anticipated that residual amounts of these compounds may have been present in these tissues. However, the presence of ERC in the lung, spleen and heart of both P. multocida and P. hemolytica infected but not control mice is not as clearly explained. Nakoneczna et al., (1969) concluded that increased levels of indole were present in the general circulation when there was either increased formation in the digestive tract or when there was a decreased rate of detoxification by the liver. An increased indoluria is present in the human in a variety of conditions, including lobar pneumonia, severe alcoholic intoxication, liver atrophy, congestive heart failure, pulmonary tuberculosis, diabetes mellitus, palegra, pregnancy toxemia and chronic arthritis (Forbes

and Neale, 1935). A possible explanation for the presence of ERC in these other tissues in infected but not control mice may be that liver, kidney, or other tissue damage impaired detoxification and elimination or increased absorption so that there were increased levels in the circulation and subsequently in tissue.

The possibility remains that higher levels of indole were present in P. multocida infected mice than in P. hemolytica or control mice. However, restrictions on the ability to detect and identify these compounds by GLC precluded this determination. The Ehrlich tube test was able to detect ERC at a minimum level of 25 ng/gm of tissue, which was 20 times as sensitive as detection levels by TLC and 4 times as sensitive as the best expected sensitivity in GLC (Bradley and Carlson, 1974). References in regard to previously utilized techniques to extract indolic compounds from tissue were not found, therefore, those techniques which had been described for extraction from plasma were used. Plasma extracted in this manner contained very few compounds which resolved on gas-liquid chromatography (GLC). However, tissue extracts contained many. Attempts to adsorb these interfering compounds were not successful. When utilizing Unisil in column chromatography, interfering compounds were removed together with Ehrlich reacting compounds. Adsorption of extracts with Florisil did not remove the ERC but also did not remove sufficient interfering compounds to an extent that GLC analysis was possible. Attempts to utilize Unisil as an adsorbent as was done with Florisil were not conducted because of a temporary commercial inavailability.

The microorganism present in intestinal flora of most animals which produces indolic compounds from tryptophan is E. coli. The

lack of large numbers of this microorganism in the fecal material of rabbits as indicated by this study and the literature (Smith, 1965) provides an explanation of the inability to detect ERC in rabbit tissue extracts. In contrast, mouse fecal material contained large numbers of E. coli and it is probably that ERC present in mouse tissue extracts were of intestinal origin.

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DEDICATION

To my wife Habiba and to our children,
Aishatu, Amina, Murtala, Mairamu, Hadiza
and Alhassan.

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INDOLIC COMPOUNDS IN TISSUES OF MICE AND RABBITS
INFECTED WITH PASTEURELLA MULTOCIDA
AND PASTEURELLA HEMOLYTICA

by

MUHAMMAD ZAIYANU ABDULLAHI

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ABSTRACT

Pasteurella multocida is the etiological agent of fowl cholera and bovine hemorrhagic septicemia. It is also one of the causative agents of rabbit septicemia, mastitis of cattle and sheep; and pneumonia of cattle, swine, sheep and goats. Humans usually become infected from animal bites and other diseases which include meningitis, empyema and bronchioectasis. Although diseases caused by this microorganism are of economic and public health significance, the mechanism by which P. multocida produces disease is poorly understood.

Pasteurella multocida produces indole in vitro. Indole and the related compound, 3-methylindole (3 MI) are lipophilic. They both cause arthritis when injected into knee joints of rabbits and hemolyse erythrocytes. Three-methylindole causes acute bovine pulmonary emphysema in cattle, sheep and goats.

One group of mice was inoculated with P. multocida intraperitoneally, another infected with P. hemolytica by the same route and a final group left as controls. Likewise, two groups of rabbits were infected conjunctivally with P. multocida and P. hemolytica respectively. Control rabbits received brain heart infusion broth (BHI) by the same route. Liver, spleen, heart, lung and kidney from each animal was collected. Similar organs in each group of mice were pooled. Plasma samples from rabbits were also collected at intervals. The organs were homogenized, extracted with methylene chloride, adsorbed with Florisil and concentrated. Pasteurella multocida and P. hemolytica broth cultures as well as BHI were treated as described for organs. Plasma samples were not treated with Florisil. The extracts were

tested with Ehrlich's reagent in tubes for the presence of indole reacting compounds (ERC) and analyzed by thin layer chromatography (TLC) and gas-liquid chromatography (GLC) for the detection of indole and 3 MI. Fresh fecal materials from both mice and rabbits were cultured for the estimation of Escherichia coli numbers.

No rabbit tissues or plasma extracts contained ERC. On the other hand, all the mice organ extracts, except normal spleen, heart and lung, were Ehrlich positive. Analysis of the samples by GLC was not successful due to presence of interfering compounds. No extracts from either rabbits or mice contained sufficient levels of ERC to be detected by TLC. Fecal samples of mice contained large numbers of E. coli, while only two colonies of this microorganism were observed in rabbit fecal culture.

Results tended to indicate that P. multocida did not produce indole or 3 MI in mice or rabbits during infection. The presence of ERC in mouse tissue extracts was probably of intestinal origin since the fecal material of this animal contained large numbers of E. coli.