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HEMAGGLUTINATION AND ADHESIVE PROPERTIES OF *Escherichia coli* ISOLATED FROM DISEASED TURKEYS

By

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ABSTRACT

HEMAGGLUTINATION AND ADHESIVE PROPERTIES OF *Escherichia coli* ISOLATED FROM DISEASED TURKEYS

By

Srikandi Fardiaz

This investigation was carried out to isolate and characterize strains of *E. coli* from diseased turkeys, and to study the hemagglutination activities (HA) and the adhesive properties of the isolates with respect to turkey small intestinal tissue. Hemagglutination and *in vitro* adhesion tests were done using unheated cultures and cultures that had been heated at 65°C for 30 minutes. The ability of various sugars and sugar derivatives, concanavalin A, sodium metaperiodate, and isolated pili to act as inhibitors of the *in vitro* adhesion was also studied.

The strains of *E. coli* isolated from diseased turkeys represented three serotypes, O1:K1, O2:K1 and O78:K80. The cultures tested possessed a mannose-sensitive hemagglutinin. Cultures 9 and 119-3 exhibited the HA type III (NNSSS), while culture 94-5 exhibited the HA type IV (SNSSS). Growth at 18°C and heating the cultures at 65°C

for 30 minutes did not affect the hemagglutination activities, but these treatments affected the adhesive properties. During incubation at room temperature the numbers of bacteria adhering to turkey small intestinal tissue increased by ca. 1.25 to 1.75 log cycles for cultures 9 and 119-3, and by ca. 1.0 log cycle for culture 94-5.

The presence of D-mannose and α-methyl-D-mannoside at 0.4 and 0.5 percent, respectively, reduced the numbers of bacteria adhering to the intestinal tissue by 1.0 log cycle. Adhesion was completely inhibited by concanavalin A and sodium metaperiodate at concentrations of 0.2 and 0.1 percent, respectively. While isolated pili were less inhibitory than D-mannose, α-methyl-D-mannoside, concanavalin A, and sodium metaperiodate, adhesion was substantially inhibited by isolated pili at a concentration of 2.0 mg protein per ml. The approximate molecular weight of protein from the isolated pili was 34,800.

To my husband, Dedi,
and my daughter, Miri

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

INTRODUCTION

Escherichia coli is a lactose-fermenting fecal bacterium in the family Enterobacteriaceae. It is a small gram-negative rod, aerobic and facultatively anaerobic, either nonmotile or motile with peritrichous flagella. *E. coli* is one of the pathogenic organisms which causes heavy losses in the poultry industry. Certain serotypes of *E. coli* have been reported to be associated with diseases in poultry (Sojka and Carnaghan, 1961; Harry, 1964; Hemsley and Harry, 1965; Heller and Smith, 1973). Pathogenicity of *E. coli* in humans and animals is associated with the ability of the bacteria to adhere and colonize in the small intestine. Adhesion of *E. coli* to intestinal epithelial cells is suggested to be mediated by pili (Jones and Rutter, 1972; Isaacson *et al.*, 1977, 1978; Moon *et al.*, 1977; Nagy *et al.*, 1977). Pili also adhere to red blood cells and cause hemagglutination (Duguid *et al.*, 1955).

Most enterotoxigenic strains of *E. coli* (ETEC) isolated from humans and animals have been found to possess specific pilus antigens, which are also mannose-resistant (MR) hemagglutinins (Jones and Rutter, 1974; Burrows *et al.*, 1976; Evans *et al.*, 1977; Evans and Evans, 1978).

These pilus antigens, which enable the bacteria to attach to the epithelial cells and colonize in the small intestine, are considered to be one of the primary determinants of virulence. However, some enterotoxigenic strains from neonatal pigs do not produce any of the known MR hemagglutinins (Moon et al., 1980), and some human enteropathogenic E. coli (EPEC) produce only mannose-sensitive (MS) hemagglutinins (Evans et al., 1979). A study by Duguid et al. (1979) showed that many strains of E. coli possessed both MS and MR hemagglutinins.

The adhesion of some E. coli strains to human and animal cells has been studied. It has been found that bacterial binding can occur via pili to the carbohydrate residues of the glycoproteins on mammalian cell surfaces (Jones and Freter, 1976; Salit and Gotschlich, 1977; Ofek et al., 1977, 1978). This study was carried out to observe the hemagglutination and adhesive properties of E. coli strains (that possessed pili) which were isolated from diseased turkeys in Michigan. The hemagglutination patterns of the E. coli strains to human and animal red blood cells, the characteristics of the in vitro adhesion of E. coli to turkey small intestinal tissue, and the inhibitory effects of certain carbohydrates on the in vitro adhesion, were determined. The inhibition of adhesion was also tested using isolated pili as an inhibitor.

LITERATURE REVIEW

Pili

Pili are nonflagellar filamentous appendages that are produced by many gram-negative bacteria. The name pili (=hair) was proposed by Brinton (1959), whereas the Latin name fimbriae (=fibers) was given to such structures by Duguid *et al*. (1955). The classification of pili has been based on their electron microscopy, function, and the hemagglutination of human and animal red blood cells by bacteria possessing the pili. Brinton (1965) distinguished six types of pili (1 to 5, and F pili), and Duguid *et al*. (1966) named F pili and six types of common pili in gram-negative bacteria. Ottow (1975) differentiated six groups of pili and four subtypes within the first group. F pili are sex pili which function in the transport of nucleic acid during bacterial conjugation and male-specific phage infection.

Pili as Adhesive Factors

The type 1 pili, found in most strains of *E. coli* are responsible for adhesion of bacteria to fungal, plant, and animal cells, including red blood cells, and epithelial

cells of the alimentary, respiratory, and urinary tracts (Duguid, 1968). Type 1 pili are also associated with the pellicle-forming property (Duguid, 1968; Old et al., 1968; Ottow, 1975). Type 1 pili from different E. coli strains are closely related (Nowotarska and Mulczyk, 1977). Further classification of pili could be done according to their distinctive physical, chemical, functional, and antigenic characteristics. Pili of types 2 to 6 are distinguished partly by their adhesiveness or lack of adhesiveness for a particular surface (Duguid, 1968).

Pilus Antigens

The groups of pili which facilitate intestinal colonization by ETEC in humans and animals consist of several antigenic types. These pilus antigens can be used in detecting ETEC infections because they occur more commonly in ETEC than in non-ETEC. One of the pilus antigens present in E. coli is the K88 antigen, described by Ørskov et al. (1964) and Stirm et al. (1967) in enterotoxigenic strains isolated from swine with diarrhea. This antigen is plasmid determined (Ørskov and Ørskov, 1966). Another pilus antigen is the K99 antigen, found in E. coli strains isolated from newborn calves and lambs with diarrhea (Ørskov et al., 1975; Burrows et al., 1976; Guinée et al., 1976; Smith and Huggins, 1978), and also in pigs (Moon et al., 1977). A pilus antigen known as 987P, which does not agglutinate animal red blood cells, has been demonstrated also in

strains isolated from pigs (Isaacson et al., 1977; Nagy et al., 1977). Some ETEC have been found that are enteropathogenic for neonatal pigs but do not produce K88, K99 or 987P (Moon et al., 1980). Two different pilus antigens known as colonization factor antigens, CFA/I (Evans et al., 1975, 1977, 1978; Ørskov and Ørskov, 1977), and CFA/II (Evans and Evans, 1978; Evans et al., 1979), have been detected in some human enterotoxigenic strains. Another pilus antigen, called F7 antigen (Ørskov et al., 1980), was found in a strain of E. coli isolated from human urinary tract infections.

Adhesive Properties of E. coli

Adhesion to Human and Animal Cells

Adhesion to human and animal cells is a factor in the pathogenicity of E. coli. Pathogenicity is the ability of an organism to cause disease in animal or plant hosts. To produce disease a pathogenic organism must accomplish the essential steps: enter the host, multiply in the host, resist or do not stimulate host defenses, and damage the host. The process in each step is complex, therefore, several determinants are usually involved in the overall process. Lack of one determinant may result in attenuation of a strain. It means that a component essential for virulence may also be present in a strain that is attenuated due to lack of some other determinants (Smith and Pearce,

1972). The determinants of virulence may not be expressed completely by microorganisms grown *in vitro*. It has been recognized that bacterial virulence may be maintained or increased during growth *in vivo*, but can be lost on subculture. The conditions of growth *in vivo* may influence the production of components necessary for pathogenicity (Smith, 1964; Smith and Pearce, 1972).

Most infections caused by microorganisms begin on the mucous membranes of the respiratory, alimentary, and urinary tracts. These membranes are protected by moving lumen contents, surface mucus, and often by commensal microorganisms (Smith, 1976; Smith and Pearce, 1972). There are at least three types of early attack on mucous membranes: adhesion and multiplication without significant penetration such as in cholera; adhesion and penetration to mucosal cells in which they multiply without or with little spread from the initial site such as in bacillary dysentery; or, adhesion and penetration into the underlying tissues either through or between the mucosal cells such as in salmonellosis, streptococcal infections, and amoebic dysentery (Schlesinger, 1975).

Certain strains of *E. coli* can cause disease in humans and animals, either by producing a cholera-like enterotoxin or by invading the intestinal epithelium. ETEC adhere and colonize the small intestine and elaborate an enterotoxin. The toxin(s) can be a heat-stable toxin (ST) that resists heating at 100°C for 15 minutes, a heat-labile

toxin (LT) that is antigenic and destroyed at 60°C for 30 minutes, or both types of toxins (Gyles and Barnum, 1969); Gyles, 1971). The strains which produce enterotoxin(s) do not invade, but the toxins they produce cause secretion of electrolytes and water into the lumen. This results in mild to severe diarrhea, and finally dehydration and shock without fever (Smith and Gyles, 1970; DuPont et al., 1971). The invasive or septicemic strains of E. coli have the ability to penetrate cells of the intestinal mucosa and cause a febrile illness with chills, fever, headache, myalgia, abdominal cramps, and watery diarrhea. The invasive strains can also cause hypertension, systemic toxemia, and tenesmus, and the feces sometimes contain blood, mucus, and abnormal numbers of epithelial cells (DuPont et al., 1971). The infections of neonatal animals characterized by bacteremia or septicemia usually are caused by the invasive E. coli strains, while the less severe diarrheic infections are caused by ETEC. Some E. coli strains, such as those found in urinary tract infections, have been called opportunistic pathogens because they are generally harmless in their normal habitat, but when they invade other tissues they can cause disease (Carter, 1976).

Factors Affecting Adhesion

The adhesion of bacteria is promoted by surface components. These surface components, primarily suggested

to be the pili, can contribute to mucosal surface infection by penetration and damage of epithelial cells, or by breaching the epithelial surface to facilitate spread of infection to other tissues (Schlesinger, 1975). Factors which affect adhesion and colonization of microorganisms are: resistance to mechanical flushing action of moving lumen contents, competition with the surface commensals for space on the mucosa and for food materials, resistance to antimicrobial materials, and resistance to humoral and cellular antimicrobial mechanisms in the mucous secretions (Smith and Pearce, 1972).

Pathogenic organisms adhere to mucous membrane surfaces with selectivity. Specific interactions between surface components of bacteria and host are responsible for the selective adherence. The specificity consists of host specificity which is the ability of microorganisms to attack some animal species in preference to others, and tissue specificity which is the ability of microorganisms to attack some tissues in preference to other tissues (Smith, 1972, 1976). The most important host influences are the nutritional environment, the nature and strength of the humoral and cellular defense mechanisms, the host receptors for initial attachment, and the barriers for spreading (Smith and Pearce, 1972). Some of the specific surface components of bacteria which are responsible for the selective adhesion and have been demonstrated to be important for virulence include K88 antigen (Jones and Rutter,

1972; Sellwood et al., 1975; Nagy et al., 1978) in E. coli strains found in piglets, K99 antigen in strains isolated from calves, lambs (Burrows et al., 1976; Smith and Huggins, 1978) and pigs (Moon et al., 1977), 987P antigen in strains isolated from pigs (Isaacson et al., 1977; Nagy et al., 1977), and CFA/I (Evans et al., 1975) and CFA/II (Evans et al., 1978) in strains isolated from human.

The probable sites for bacterial adhesion and subsequent colonization are the brush borders of the epithelial cells and the overlaying mucous membrane (Jones, 1975). Brush border surfaces are covered with a carbohydrate-rich layer, called glycocalyx (Ito, 1969). The glycocalyx is composed primarily of various glycoproteins (Forstner, 1971). Bacteria probably adhere to the sugar residues of the glycocalyx. Ofek et al. (1977, 1978) described the inhibitory effects of D-mannose and methyl-α-D-mannopyranoside in the adhesion of E. coli to epithelial cells of the human oral cavity, and Salit and Gotschlich (1977) described the inhibitory effects of the same sugars in the adhesion of E. coli to monkey kidney cells. Yeast mannan, a polymer of D-mannose, is also a strong inhibitor of adhesion (Ofek et al., 1978). These reports suggest that saturation of binding sites on the bacterial surface by a sugar prevents the attachment of these organisms to the epithelial membrane receptors which contain D-mannose or a mannose-like structure. A study by

Jones and Freter (1976) found that L-fucose, and to some extent D-mannose, inhibited the adhesion of Vibrio cholerae to rabbit intestinal epithelial cells, but the hemagglutination activity was inhibited only by L-fucose, not by D-mannose. The absence of hemagglutination inhibition by D-mannose appeared to distinguish the hemagglutination activity from adhesion to epithelial cells.

Hemagglutination (HA) Activities of E. coli

Mannose-Sensitive (MS) HA

Three groups of E. coli strains were distinguished by Duguid et al. (1955) based on their patterns of hemagglutination activity against red blood cells of different animal species. The HA activity of group I strains is associated with the presence of type 1 pili (Duguid et al., 1955; Duguid, 1968; Ottow, 1975). Their HA activity is best developed in stationary-phase cultures in static liquid media (Duguid et al., 1979). Some of these E. coli strains do not form the type 1 pili when grown on a solid medium (Ørskov et al., 1980). The HA activity of type 1 pili is inhibited by 0.5 percent (w/v) D-mannose and methyl-α-D-mannoside (Duguid and Gillies, 1957). According to Duguid et al. (1979), the MS hemagglutination persisted when the test mixture was warmed to 50^{o}C in the absence of D-mannose, and the HA activity was unaffected when the bacteria were heated at 65^{o}C for 30 minutes.

Mannose-Resistant (MR) HA

The HA activities of groups II and III strains are resistant to inhibition by D-mannose (Duguid et al., 1955; Duguid and Gillies, 1957). Group II is different from group III in that group II strains possess pili, while group III strains do not (Duguid et al., 1955). The HA activity of the MR groups is destroyed when the bacteria are heated at 65°C for 30 minutes (Duguid et al., 1979). The reactions between some MR strains and red blood cells of some animal species develop only in the cold (3-5°C), and the HA activities elute when the test mixtures are warmed to 20°C. When an eluted mixture is cooled again to 3-5°C, it will reagglutinate. However, most MR strains agglutinate red blood cells at 20°C, and the reaction elutes when the test mixture is warmed to 30, 40 or 50°C (Duguid et al., 1955). The hemagglutinins, which elute when warmed to 30-50°C, were called mannose-resistant and eluting (MRE) hemagglutinins by Duguid (1964). The MRE hemagglutinins are best developed in cultures grown on agar at 37°C, but not at temperatures below 25°C (Duguid et al., 1979). Jones (1977) proposed the name "fibrillae" for the MRE-type pili.

The HA properties of group II strains have been found to be associated with enteropathogenicity of E. coli and the presence of pilus antigens such as K88 (Jones and Rutter, 1974; Isaacson et al., 1977), K99 (Ørskov et al.,

1975; Guinée et al., 1976; Burrows et al., 1976), CFA/I (Evans et al., 1975, 1977, 1979; Ørskov and Ørskov, 1977), CFA/II (Evans and Evans, 1978; Evans et al., 1979), and F7 (Ørskov et al., 1980). Although the HA activities of these pilus antigens with different species of animal red blood cells are resistant to D-mannose, HA activities of some CFA/I-positive strains with guinea pig red blood cells and HA activities of some CFA/II-positive strains with human and guinea pig red blood cells are sensitive to D-mannose (Evans et al., 1979).

Many strains of E. coli possess both MS and MRE hemagglutinins (MS^+/MRE^+), and it is usually impossible to identify separately the two types of pili by electron microscopy. The presence of MRE hemagglutinin in an MS^+/MRE^+ strain, according to Duguid et al. (1979), could be detected by its positive reactions with red blood cells of some animal species in the absence and presence of D-mannose. The MS hemagglutinin in such strain could be detected by warming the test mixture to 50°C, at which the hemagglutination persisted in the absence of D-mannose, but eluted in the presence of D-mannose. Another way to detect the MS activity in such strains is by testing with a culture that has been heated at 65°C for 30 minutes to destroy its MRE hemagglutinin. Based on these methods, E. coli strains were classified by Duguid et al. (1979) into four types: MS^+/MRE^+, MS^+/MRE^-, MS^-/MRE^+, and MS^-/MRE^-. Evans et al. (1979) developed the HA patterns of human enterotoxigenic

and enteropathogenic E. coli, and classified them into four types (types I to IV) based on the MS, MR, and negative (N) hemagglutination with human, bovine, chicken, and guinea pig red blood cells.

Nature, Formation, and Isolation of Pili

Nature and Composition of Pili

Like flagella, pili originate from the cytoplasma and penetrate through the peptidoglycan layers of the cell wall (Hoeniger, 1965). Pili are distinguishable from flagella under the electron microscope by their smaller diameter, irregular length, and lack of a wave-like motion (Brinton and Stone, 1961). However, pili are reported to have a rod shaped, rigid, helical structure similar to that of bacterial flagella (Brinton, 1965). Different cells from one culture may carry from one up to a thousand pili (Brinton, 1965; Duguid, 1968). Brinton et al. (1964) described the type 1 pili of E. coli as having an external diameter of 7 nm and an internal diameter of 2.0-2.5 nm. The K99 pili have a diameter of 7.0-9.8 nm, and a length between 84 and 183 nm (Isaacson, 1977), while the pili purified by Korhonen et al. (1980) have a diameter of 5-7 nm, and a length between 0.5 and 1.0 μm.

Studies with pure pili have shown that E. coli pili consist of nearly 100 percent protein. These proteins are

called pilin, and contain less than 0.6 percent each of DNA, RNA, phosphate, polysaccharides, and reducing sugars (Brinton and Stone, 1961). Stirm et al. (1967) purified the K88 pilus antigen, and found that K88 was a pure protein which had a sedimentation coefficient ($S^{o}_{20,w}$) of 36.7S. This protein contained all the common amino acids except for cysteine and cystine. The K88 pilus antigen is a K antigen of the thermolabile L types because the protein denatures at temperatures above 70°C.

A study by Isaacson (1977), which used SDS-gels for the determination of the molecular weight of the K99 pilus protein, showed that there were two different bands from K99 protein, one had a molecular weight of 22,500, and the other had a molecular weight of 29,500. When the gels were scanned at 550 nm, the results indicated that there were five subunits in the first band and the other band was homogeneous. Less than 0.6 percent neutral sugars and 6.6 percent lipid were detected in K99 protein. The K99 protein contained few aromatic amino acids, with no tyrosine or phenylalanine residues. Isoelectric focusing showed that K99 protein had a pI of 4.2 (Morris et al., 1978).

The type 1 pili of a substrain of E. coli K-12 were reported to consist of subunits with apparent molecular weights of 15,500, 17,000, or 19,000, depending on the degree of separation and age of preparation (McMichael and Ou, 1979). The pili purified by Korhonen et al. (1980) from E. coli strains isolated from patients with urinary

tract infections had a single subunit with a molecular weight of 17,000, a pI of 4.9, and 43 percent of the amino acids in the subunit protein were hydrophobic.

Methods for Isolation of Pili

Separation of pili from the bacterial cells is easily achieved by blending of cultures in the logarithmic growth phase (Brinton and Stone, 1961; Brinton, 1965; Novotny et al., 1969). Novotny et al. (1969) reported that blending at a speed of 4,220 rpm for 2 minutes was effective in removing flagella and F pili of E. coli, but not type 1 pili, while blending at 11,650 rpm for 2 minutes completely removed type 1 pili. The extracted pili can be precipitated with ammonium sulfate (Brinton, 1959; Isaacson, 1977; McMichael and Ou, 1979; Korhonen et al., 1980) or by using ultracentrifugation (Stirm et al., 1967; Evans et al., 1978; Freer et al., 1978). If the sediment from the ultracentrifugation is suspended in a neutral solution and left for several days at $4^{o}C$, the aggregated pili can be separated from the remaining impurities by centrifugation at a higher speed (Brinton and Stone, 1961). Further purification of pili can be accomplished by using gel filtration chromatography (Isaacson, 1977; Korhonen et al., 1980), ion exchange gel chromatography (Isaacson, 1977), or ultracentrifugation in sucrose gradients (Isaacson, 1977; Evans et al., 1978; Korhonen et al., 1980).

MATERIALS AND METHODS

Sources of E. coli Isolates

E. coli isolates were obtained from Bil-Mar Foods Inc., Zeeland, Michigan, from diseased turkeys during several E. coli outbreaks that occurred in 1978 and 1979. The E. coli outbreaks occurred in young turkeys which were three to five weeks old. During these outbreaks between 10 and 100 birds per day were lost in houses containing approximately 10,000 birds. E. coli isolates were also obtained from normal turkeys, feed, water, and litter in August 1979.

Isolation of E. coli

Plates were inoculated by a veterinarian using swabs from different parts of the body of diseased turkeys such as lung, liver, heart, spleen, and yolksac. Each plate contained three separated media, Eosin methylene blue (EMB) agar for the detection and isolation of E. coli, Brain heart infusion (BHI) agar for the cultivation of fastidious pathogenic bacteria, and Brilliant green (BG) agar which is a highly selective medium for the isolation of Salmonella. After incubation at 37°C for one day, the

plates were sent to Michigan State University.

Several colonies from each plate were streaked on EMB plates, and the plates were incubated at 37°C for 20-24 hours. Typical E. coli colonies were picked from EMB plates, and purified by repeated streaking on EMB plates. After incubation on EMB plates at 37°C for 20-24 hours, the pure cultures were then streaked onto slants of Blood agar base (BAB) which were incubated at 37°C for 20-24 hours. The slants were stored at 4°C for a short period, or inoculated into Casamino yeast extract (CYE) broth and frozen or lyophilized for long-term storage.

Biochemical Identification of E. coli

The cultures were screened based on their gram stain reaction (Frazier et al., 1968), and the results of oxidase tests using Kovacs' method (Kovacs, 1956). Oxidase-negative, gram-negative bacilli were identified using the Fisher Entero-Set^{TM}20 identification system.

The growth from a BAB slant, which had been incubated at 37°C for 18 hours, was suspended in 5 ml of 0.15M sterile saline. Water (9 ml) was added to the bottom of the incubation chamber, and a Fisher Entero-Set^{TM}20 card which consisted of 20 test capillaries was placed in the bottom of the chamber. Each capillary was filled with 5 drops of bacterial suspension using a sterile Pasteur pipette, and the plastic cover was sealed. After incubation for 24 hours at 37°C, $FeCl_3$ solution was dropped into the

PHE (phenylalanine deaminase) capillary, and Kovac's reagent was dropped into the IND (indole production) capillary. Reactions were read in the center of the capillaries, unless the capillaries were bracketed at the top. The organisms were identified using the Entero-Trak^{TM}20 manual, or using the Entero-Set^{TM}20 identification table (Table 1).

Serological Identification of E. coli

O Serotype

Preparation of O Antigen. Serological identification of O antigen was done based on the methods described by Mehlman et al. (1974) and Ørskov and Ørskov (1975). A pure culture was inoculated into a BAB slant and incubated at 37^{o}C for 24 hours. Using the growth from the BAB slant, a suspension was prepared in 15 ml of 0.15M saline with turbidity corresponding to McFarland standard number 3 (9.0 x 10^{8} cells/ml). The suspension was boiled at 100^{o}C for one hour (to inactivate the capsular antigen), cooled, and 0.07 ml of 37 percent formaldehyde (0.5 percent formalin) were added as a preservative.

Presumptive Identification. The O antisera used were obtained from Difco Laboratory (Detroit, Michigan), and represent the O antigens of E. coli strains found in diseased poultry, i.e. O1, O2, O8 and O78 (Sojka and Carnaghan, 1961; Harry, 1964; Hemsley and Harry, 1965; Heller and Smith, 1973). The presumptive identification of O antigens

Table 1. Identification of *E. coli* by Fisher Entero-Set™20

Test	Description	Typical *E. coli* reaction	Entero-Set™20	
			No.	Total no.[a] (typical *E. coli*)
MAL	Malonate utilization	-	1	
GLU	Glucose-Resazurin	+	2	2
PHE	Phenylalanine deaminase	-	4	
ONPG	ONPG-beta-galactosidase	±	1	
IND	Indole production	+	2	2 or 3
H_2S	H_2S production	-	4	
LYS	Lysine decarboxylase	±	1	
ORN	Ornithine decarboxylase	±	2	0, 1, 2 or 3
UR	Urease	-	4	
SUC	Sucrose fermentation	±	1	
ARG	Arginine dihydrolase	±	2	0, 1, 2 or 3
CIT	Citrate utilization	-	4	
SAL	Salicin fermentation	±	1	
AD	Adonitol fermentation	-	2	0 or 1
IN	Inositol fermentation	-	4	
SOR	Sorbitol fermentation	+	1	
ARB	Arabinose fermentation	+	2	3 or 7
MLT	Maltose fermentation	±	4	
TR	Trehalose fermentation	+	1	1 or 3
XY	Xylose fermentation	±	2	

[a]Total number represents the total number of positive reactions.

by tube agglutination was done by adding into a small tube 0.125 ml 0.15M saline, 0.125 ml monovalent O serum (1:10), and 0.250 ml culture suspension. After mixing, the tube was incubated at 37°C for 24 to 48 hours. A tube which contained 0.250 ml 0.15M saline and 0.250 ml culture suspension served as a control for autoagglutination. A negative agglutination reaction in the control tube and a positive agglutination reaction in the other tube containing a specific O antiserum (the minimum titer was 40) indicated a positive presumptive reaction. Cultures which showed negative presumptive reactions were autoclaved at 120°C for 2 hours, and tested again for presumptive identification. The autoclaved cultures were discarded if they still had not shown any positive agglutination reaction with the above antisera.

Determination of O Titer. A row of 10 unscratched 13x10 mm tubes were aligned in a rack, and 0.5 ml 0.15M saline was added to all tubes. Then 0.5 ml of the monovalent O antiserum (1:10) showing a positive presumptive reaction was added to the first tube, mixed, and 0.5 ml from the first tube was transferred to the second and mixed. Dilutions were continued through 9 tubes, and 0.5 ml from tube 9 was discarded. Tube 10 served as a control for autoagglutination. Then 0.5 ml of culture suspension was added to each tube and mixed by gentle agitation, so that the first tube contained a 1:40 final dilution and tube 9

contained a 1:10,240 final dilution of the antiserum. The O-tube agglutination reaction was read after 24 to 48 hours at 37°C. Many cross-reactions might exist between O antigens, therefore a culture suspected to belong to a given O serotype should give a titration value similar to, or higher than, the known control culture. The cultures used as controls for O antigens were U5-41 (O1:K1:H7) for O1 antigen, A20a (O2:K1:H6) for O2 antigen, G3404-41 (O8:K8:H4) for O8 antigen, and E38 (O78:K80) for O78 antigen. These cultures were obtained from Drs. I. Ørskov and F. Ørskov, Center for Reference and Research on Escherichia, Copenhagen, Denmark.

K Serotype

Immunodiffusion. Immunodiffusion was used as a screening method to detect the K serotypes of E. coli. Double diffusion was done on agarose-coated glass slides (Cahill and Glantz, 1978). The bacterial suspension was prepared using a procedure similar to that used for the identification of O antigens, with K1, K80, K88ab, K88ac, and K99 antisera purchased from the Veterinary Science Department, Pennsylvania State University. The differences in the preparation of bacterial suspension for immunodiffusion were: a Trypticase soy agar (TSA) slant was used to grow the bacteria, the bacterial suspension was heated in a water bath at 60°C for 20 minutes to release the heat-labile

antigen into the supernatant, and the heated suspension was centrifuged at 20,000 x g for 20 minutes at 4°C. The supernatant was removed and stored at 4°C. Three glass slides were coated with 11 ml of one percent agarose in Veronal buffer, pH 8.6, ionic strength 0.025 μ. Five wells were prepared, and an antiserum was placed in the center well while the antigen extracts were placed in the outer wells. The slides were stored for 4 days to two weeks in a walk-in refrigerator at 4°C, or at room temperature for 4-10 days. The results were recorded as negative or positive precipitation. The cultures used for controls of K antigens were U5-41 for K1 antigen, E38 for K80 antigen, Moon 263 (O8:K87:K88ab:H19) for K88ab antigen, G1253 (O147:K88ac:H19) for K88ac antigen, and 1474 (O^-:K99) for K99 antigen. Cultures Moon 263 and 1474 were obtained from Dr. H.W. Moon, National Animal Disease Center, Ames, Iowa, while culture G1253 was obtained from Drs. I. Ørskov and F. Ørskov.

Determination of K Titer. The determination of K titer was done as in the determination of O titer, except that the bacteria were grown on a TSA slant, the bacterial suspension was not heated, and a series of 14 tubes were used for agglutination reaction (final dilution 1:163,840).

Electron Microscopy

Cells from a culture grown on a Peptone agar slant were harvested and suspended in distilled water. Negative staining was done by floating a carbon-coated collodion grid

with a drop of cell suspension for 30 seconds, and removing the excess suspension by adsorption onto filter paper. The grid was then stained with 2 percent phosphotungstic acid for 30 seconds. After the excess of stain was removed, the grid was examined in a Philips EM 200 electron microscope to detect the presence of pili on the bacterial cells. The approximate diameter of the pili was determined by measuring the diameter of the pili from electron photomicrographs, using a micrometer (1 division = 0.01 mm). Data reported are the average of ten observations.

Hemagglutination Tests

The hemagglutination (HA) tests were done using human type A red blood cells obtained from The American Red Cross (Lansing, Michigan), and bovine, chicken, guinea pig and Rhesus monkey red blood cells obtained from Flow Laboratories Inc. (Rockville, Maryland). Red blood cells, which were preserved in Alsever's solution, were centrifuged and washed three times with 0.15M phosphate buffer, pH 7.2, containing 0.15M saline (PBS), immediately before use, and made up to a 3 percent (v/v) suspension in PBS. HA tests were carried out by a slide agglutination procedure, which was a combination of methods described by Duguid et al. (1979) and Evans et al. (1979).

Bacterial cells were obtained from cultures grown at 37°C for 18 hours or at 18°C for 72 hours, on a Colonization

factor antigen (CFA) agar slant and in CYE broth. CFA agar consists of 1 percent Casamino acids (Difco), 0.15 percent yeast extract (Difco), 0.005 percent $MgSO_4$, 0.0005 percent $MnCl_2$, and 2 percent agar, pH 7.4 (Evans et al., 1979). CYE broth consists of 2 percent Casamino acids (Difco), 0.6 percent yeast extract (Difco), 0.25 percent NaCl, 0.871 percent K_2HPO_4 (0.05M), and 0.1 percent trace salts solution, pH 8.5 (adjusted with 0.1N NaOH). The trace salts solution consists of 5.0 percent $MgSO_4$, 0.5 percent $MnCl_2$, and 0.5 percent $FeCl_3$ dissolved in 0.001N H_2SO_4 (Evans et al., 1973).

Growth from the CFA slant was suspended in PBS and growth from CYE broth was centrifuged at 17,000 x g for 10 minutes and suspended in PBS. The suspensions in PBS were adjusted to a turbidity corresponding to McFarland standard number 5 (1.5×10^9 cells/ml). One drop of bacterial suspension, unheated or heated at 65°C for 30 minutes, one drop of PBS or PBS containing 1.5 percent (w/v) D-mannose, and one drop of blood suspension were mixed on a glass slide which was placed on the surface of crushed ice. After observation for at least 2 minutes in a walk-in refrigerator (temperature 4°C) with intermittent mixing by rotation of the slide, the slide was placed at room temperature with continued rotation. Complete HA was recorded as 4+, the lesser degrees of HA were recorded as 3+, 2+, or 1+. The HA reaction was considered as a positive reaction if the reaction was $>2+$. HA was reported as mannose-

resistant (R) if the same degree of HA occurred with and without D-mannose, and as mannose-sensitive (S) if the HA was eluted or prevented in the presence of D-mannose. The persistence or elution of HA reactions at elevated temperatures was demonstrated by warming the slides to 40°C and 50°C (Duguid et al., 1979) in different incubators with intermittent rotation for no longer than 2 minutes at each temperature to prevent drying of the test mixture. Each test was done in duplicate. Culture H10407 (CFA/I^+), obtained from Drs. D.G. Evans and D.J. Evans, The University of Texas Medical School, Houston, Texas, and culture Moon 263 ($K88ab^+$) were used as controls for hemagglutination reactions.

In Vitro Adhesion Tests

The in vitro adhesion tests were done by a modification of procedures described by Jones and Rutter (1972) and McNeish et al. (1975) which used piglet small intestinal tissue. The modified procedure, outlined in Figure 1, used turkey small intestinal tissue obtained from Bil-Mar Foods Inc. The tissue preparation was freshly used, or stored for a maximum of 3 weeks at 4°C in 5 mM EDTA (pH 7.4) containing 10 percent (v/v) formalin. According to Jones et al. (1976) and Isaacson et al. (1978), a tissue extract could be stored by this method for at least 3 months after preparation, with no difference in the extent

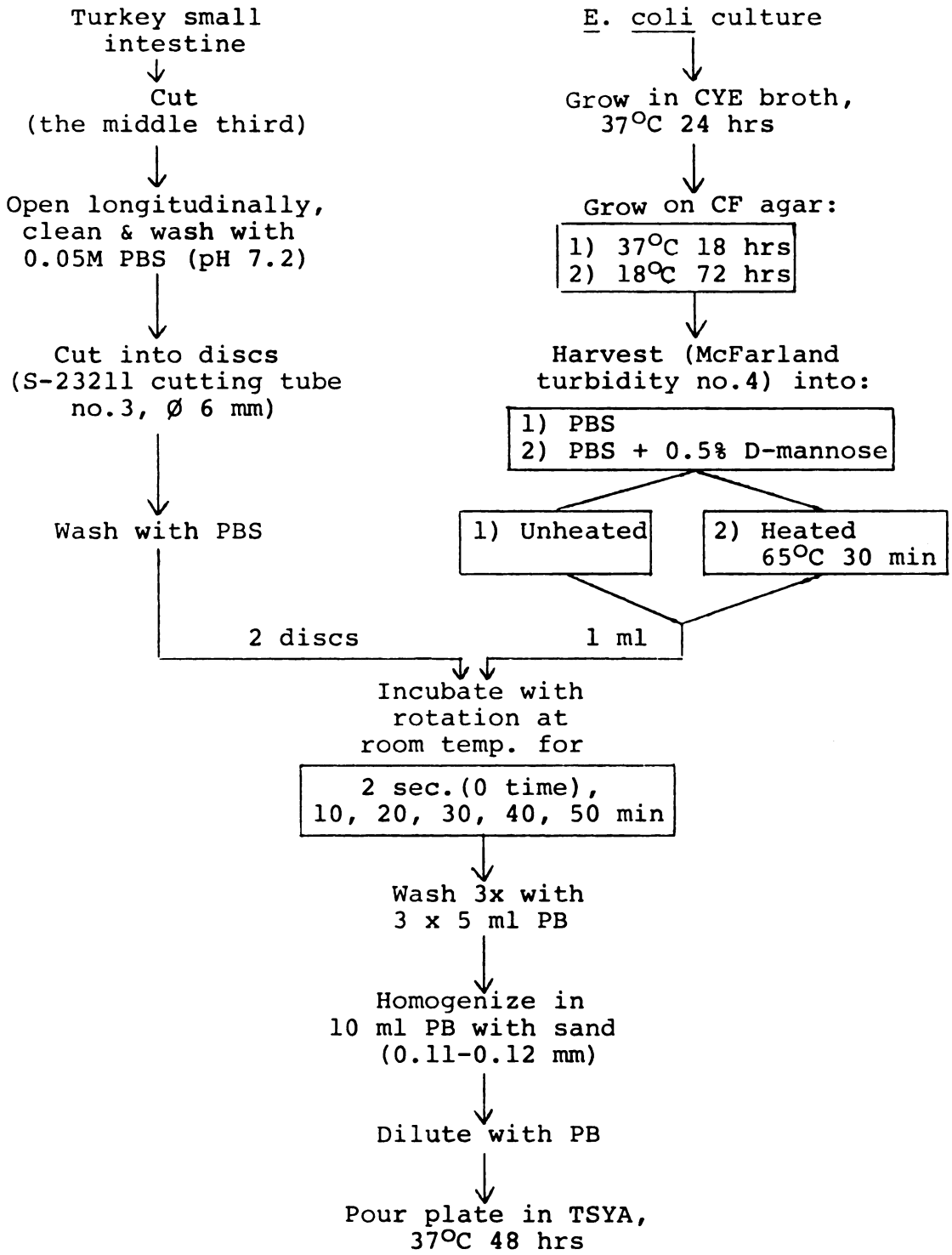

Figure 1. Flow chart of the in vitro adhesion tests.

of adhesion of bacteria between the formalin-preserved tissue and the freshly prepared tissue. However, in these experiments most of the tissues used were freshly prepared. When tested, the adhesive properties of E. coli to formalin-preserved turkey small intestinal tissue was similar to that which occurred with the freshly prepared tissue. The viable counts of the in vitro adhesion tests were determined using pour plates of Trypticase soy agar containing 0.3 percent yeast extract (TSYA) (Ray and Speck, 1973ab). The results are reported as the average of duplicate plates of four tissue samples, using two incubation tubes per treatment and two tissue samples per tube. Plate counts of the uninoculated turkey small intestinal tissues were also made to determine the number of organisms present in the original tissues.

Inhibition of In Vitro Adhesion

D-glucose, D-galactose, D-mannose, L-fucose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, N-acetyl-neuraminic acid, and α-methyl-D-mannoside (Sigma Chemicals, St. Louis, Missouri), were each used at 0.5 and 1.0 percent (w/v) concentrations to test their effects on in vitro adhesion. Six tubes were used for each sugar or sugar derivative; 0.1 ml buffer (PBS) was added to the first two tubes, 0.05 ml PBS and 0.05 ml of 10 percent (w/v) sugar solution were added to the next two tubes, and 0.1 ml of

10 percent sugar solution was added to the other two tubes. Then 0.9 ml of bacterial suspension was added to all tubes, so that each tube contained the same dilution of bacterial suspension, and the first two tubes, which served as controls, contained no sugar, the next two tubes contained 0.5 percent sugar, and the other two tubes contained 1.0 percent sugar. The next steps were the same as in the in vitro adhesion tests (Figure 1), using only an incubation time of 50 minutes. A similar procedure was also applied using D-mannose and α-methyl-D-mannoside at 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 1.5, 2.0, and 2.5 percent (w/v) concentrations.

In some experiments, concanavalin A (Sigma), sodium metaperiodate (MCB), and isolated pili were added to the intestinal tissue discs 30 minutes prior to the addition of the bacterial suspension. After this preincubation, the bacterial suspension was added to yield a final concentration of 0.05, 0.10, 0.15, 0.20, and 0.25 percent (w/v) of concanavalin A and sodium metaperiodate, and 0.5, 1.0, 1.5, 2.0, 2.5, 5.0, and 7.5 mg pilus protein per ml.

Isolation of Pili

Extraction and Precipitation

Extraction of pili from the cells was done by a method described by Novotny et al. (1969). Bacterial cells were first blended at lower speed (4,500 rpm for 2 minutes)

to break the flagella that might be present, without affecting the pili. After removal of the flagella by centrifugation at 14,350 x g for 30 minutes, the cells were blended at higher speed (12,000 rpm for 4 minutes) to break the pili, followed by centrifugation at 14,350 x g for 30 minutes to separate the pili from the cells. Precipitation of the pili was done by adding ammonium sulfate to 65 percent saturation (Isaacson, 1977). The detailed procedures for the extraction and precipitation of pili are outlined in Figures 2 and 3, respectively. The ammonium sulfate precipitate was lyophilized and stored in a freezer at $-18^{o}C$.

Gel Chromatography

The lyophilized ammonium sulfate precipitate was resuspended in 5 ml 0.05M sodium phosphate buffer (PB), pH 7.2, and applied to a Sephadex G-50 (Pharmacia Fine Chemicals) column, 2.5 by 40 cm, that had been equilibrated with PB. Blue dextran 2000 (Pharmacia Fine Chemicals) was added to the solution before chromatography as a marker to indicate the void volume (V_o), and the protein was eluted with PB at 1.5 ml per minute. The protein was detected using an Isco Model UA-2 Ultraviolet Analyzer connected to a recorder (Isco Instrumentation Specialties Co., Inc.; Lincoln, Nebraska). Fractions of 3 ml were collected using a fraction collector (Rinco Instruments Co.; Greenville,

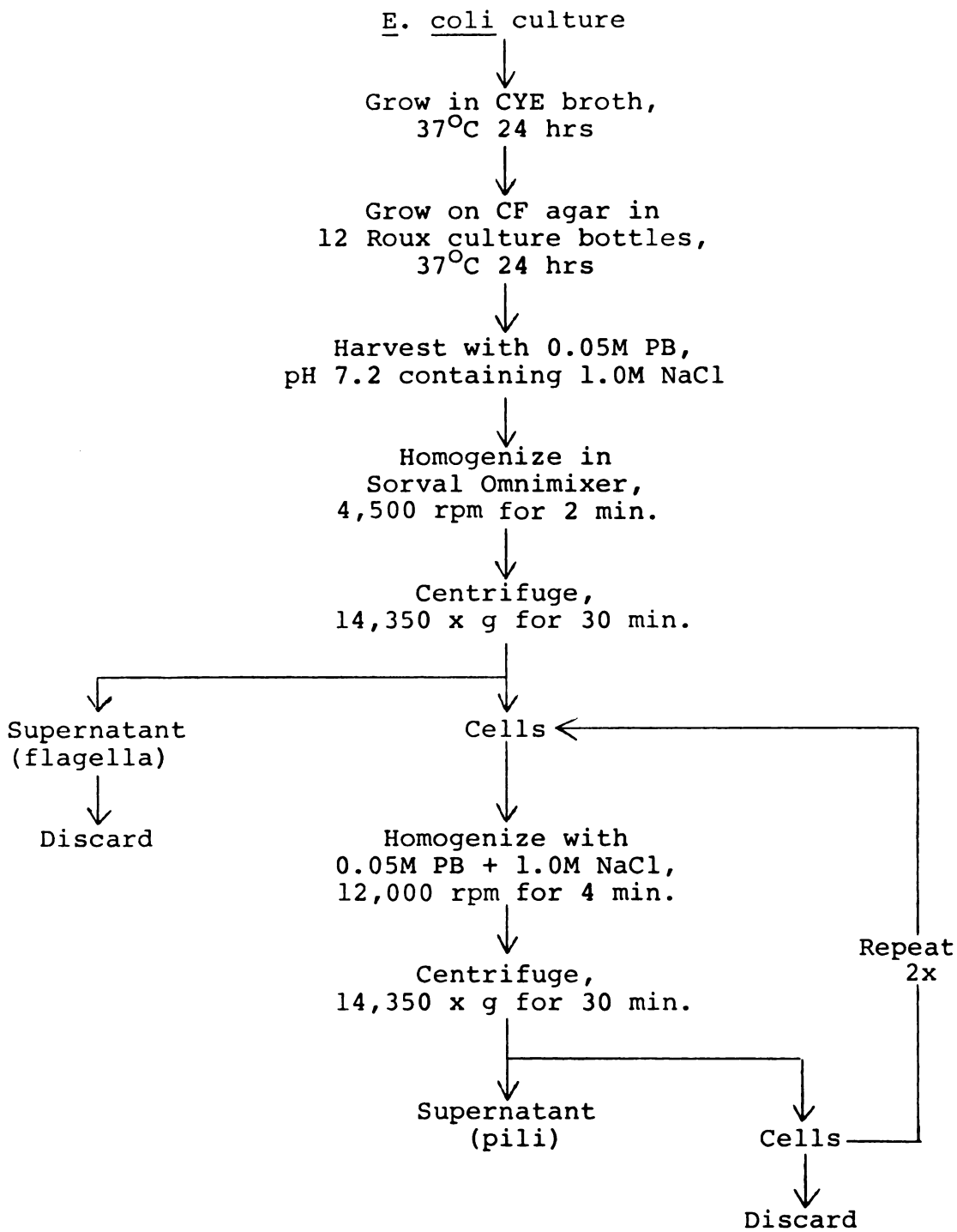

Figure 2. Flow chart of the extraction of pili from bacterial cells.

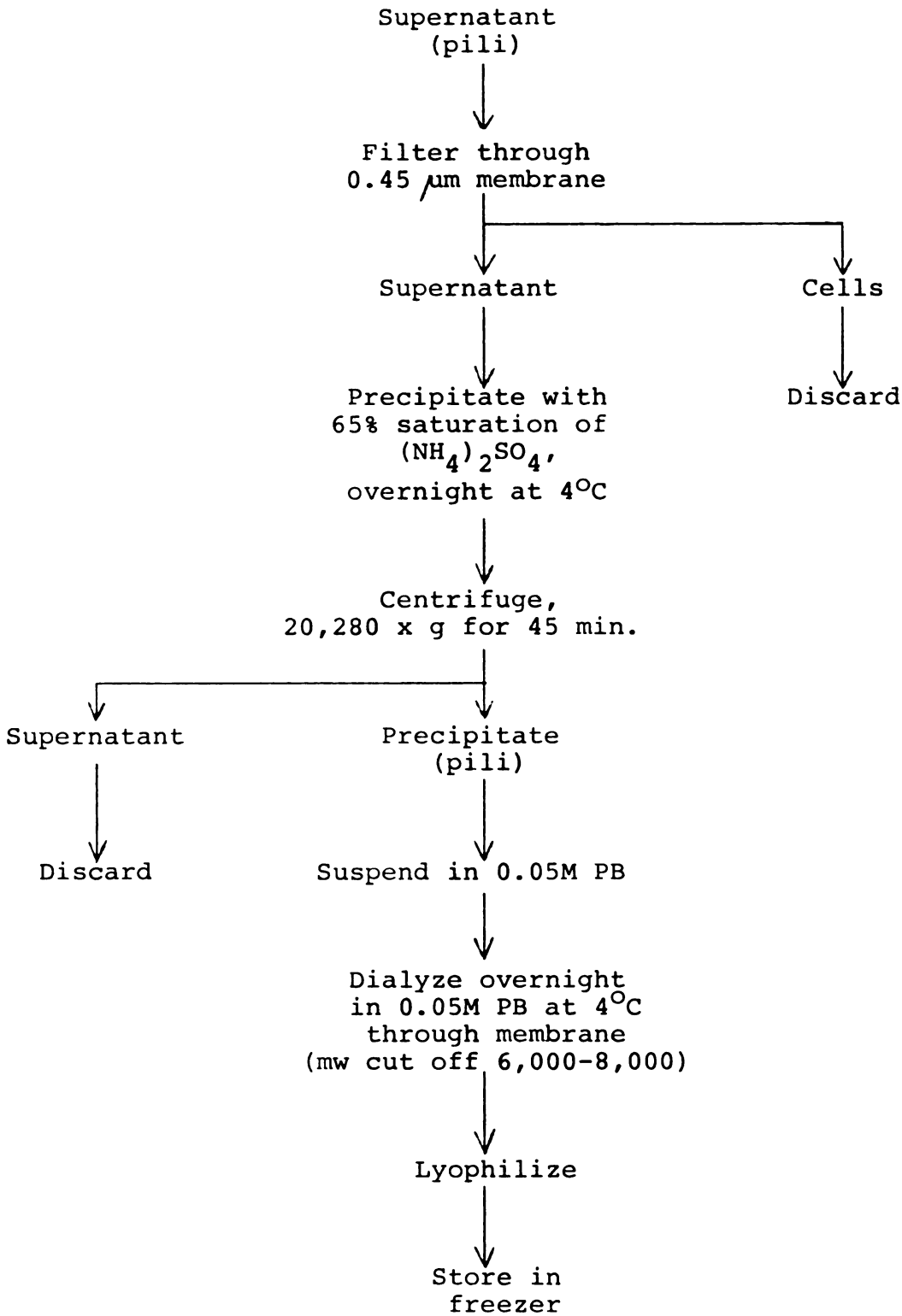

Figure 3. Flow chart of the precipitation of pili.

Illinois), and were assayed for absorbance at 280 nm (A_{280}) using a Beckman DU Model 2400 Spectrophotometer (Beckman Instruments, Inc.; Fullerton, California). Fractions which belonged to the primary peak were pooled and the mixture was lyophilized, and stored in a freezer at -18°C.

Gel Electrophoresis

The gel for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was prepared by the method of Weber and Osborn (1969) as described by Cooper (1977), using 7.5 percent acrylamide (T). The gel was prepared by mixing 15 ml gel buffer (0.2M phosphate, pH 7.2, 0.2 percent SDS), 10.1 ml acrylamide solution (22.2 g acrylamide and 0.6 g N,N'-methylene-bis(acrylamide) in a final solution of 100 ml), 3.4 ml water, 45 µl TEMED (N,N,N',N'-tetramethylethylenediamine), and 1.5 ml ammonium persulfate solution (35 mg ammonium persulfate in 10 ml water).

For electrophoresis the lyophilized sample was dissolved in buffer (0.01M phosphate, pH 7.0, 1.0 percent SDS, 1.0 percent mercaptoethanol), and the solution was heated in a boiling water bath for 5 minutes. After cooling, crystalline sucrose was added to increase the density, and a drop of 1.0 percent bromophenol blue solution was added as a marker dye.

The upper and lower chambers were filled with reservoir buffer which consisted of one part gel buffer and

one part water. Then 25 to 50 μl of 0.5 percent of the sample solution were applied to the gels. A current of 8 mA per tube was applied, and the electrophoresis was run for 4 to 5 hours in a water-cooled cell. The gels were stained in a 3.5 percent perchloric acid solution containing 0.04 percent (w/v) Coomassie brilliant blue G-250 (Sigma) for 5 hours, and destained in a 7.5 percent acetic acid solution containing 5 percent methanol. The gels were stored in 7.5 percent acetic acid.

The approximate molecular weight of the isolated pili was determined from a standard curve prepared using a mixture of proteins (Pharmacia Fine Chemicals) containing α-lactalbumin (mw. 14,400), soybean trypsin inhibitor (mw. 20,100), carbonic anhydrase (mw. 30,000), ovalbumin (mw. 43,000), bovine serum albumin (mw. 67,000), phosphorylase b (mw. 94,000), and sucrose. Relative mobilities were calculated as follows:

$$\text{Relative mobility} = \frac{\left(\begin{matrix}\text{Protein}\\ \text{distance}\end{matrix}\right) \times \left(\begin{matrix}\text{Gel length}\\ \text{before staining}\end{matrix}\right)}{\left(\begin{matrix}\text{Marker dye}\\ \text{distance}\end{matrix}\right) \times \left(\begin{matrix}\text{Gel length}\\ \text{after destaining}\end{matrix}\right)}$$

The standard curve was a plot of the relative mobilities vs log molecular weights of the standard proteins.

Quantitation of Protein

Protein concentration was determined by the method of Lowry *et al.* (1951) as described by Cooper (1977).

Absorbance at 660 nm was measured on a Spectronic 20 (Bausch & Lomb Inc.; Rochester, New York). A standard curve was developed, using bovine serum albumin (Sigma) as the standard protein. The curve was linear ($r = 0.9981$) up to 180 µg protein (Figure 4) according to the formula $y = 0.0037 x + 0.0097$, where y was absorbance at 660 nm (A_{660}) and x was the amount of protein in µg.

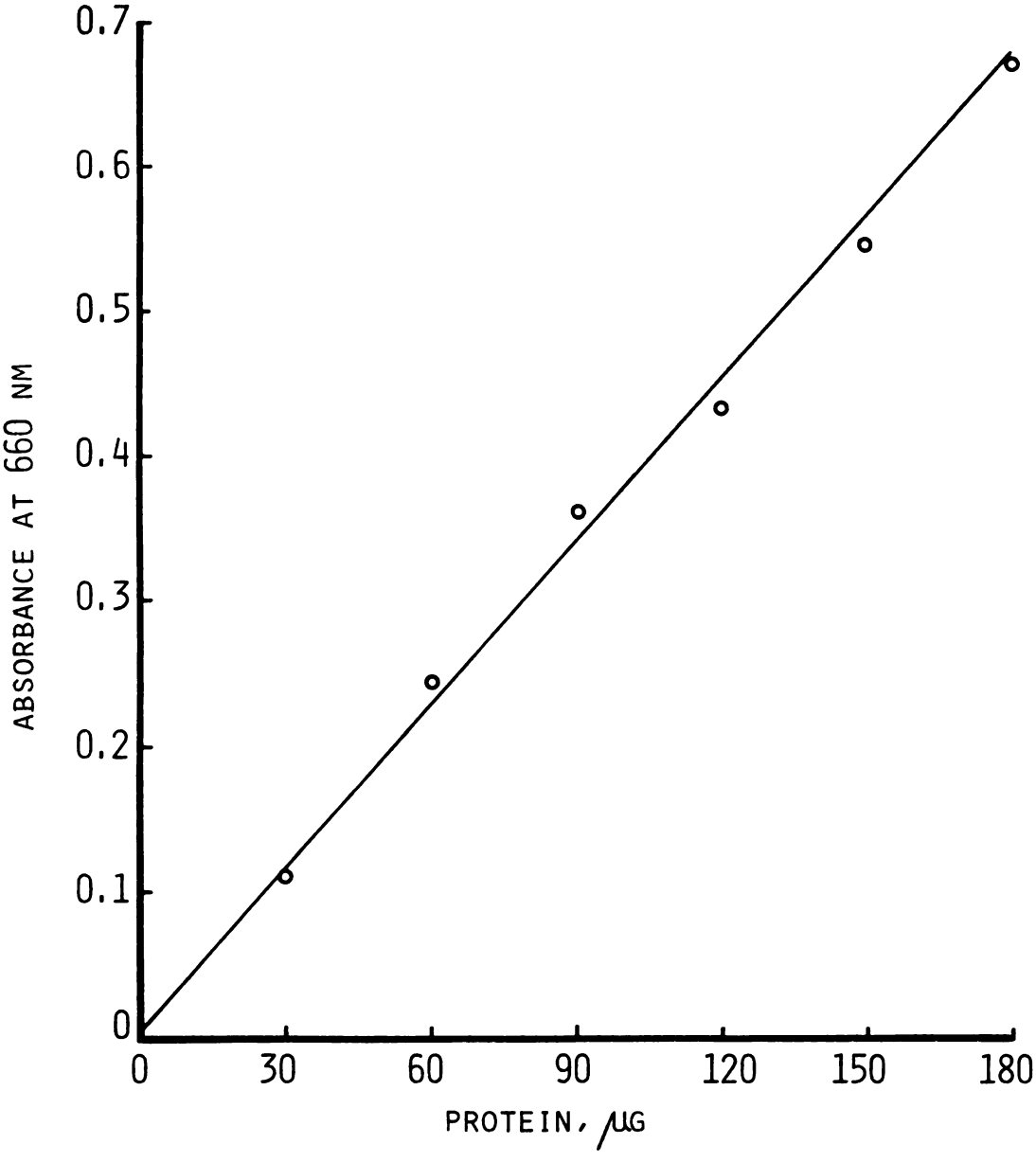

FIGURE 4. STANDARD CURVE FOR PROTEIN ASSAYED BY THE LOWRY METHOD.

RESULTS

Isolation and Identification of E. coli

Isolated Cultures

Plates from various fatal infections of poultry that occurred in 1978 and 1979 were obtained from Bil-Mar Foods Inc. (Table 2). The plates were inoculated with swabs from parts of the bodies of diseased turkeys such as lung, liver, heart, spleen, and yolksac. Due to the difficulties in differentiating between the commensal and the infectious E. coli in the intestinal tract, strains were not isolated from the intestines or feces. Preliminary data indicated that the intestines of normal young turkeys contained 10^5 to 10^6 fecal coliforms per gram, while the litter obtained from different flocks contained 10^6 to 10^7 fecal coliforms per gram.

After streaking on EMB plates, all colonies showed the typical green metallic sheen of E. coli colonies. Two different separated colonies were isolated from each plate, however, the two cultures from the same plate invariably had the same Entero-Set^{TM}20 numbers and O titers, therefore, only one culture from each plate was reported. As

Table 2. Descriptions of plates inoculated from diseased turkeys[a]

Date	Source	Condition of turkey	No. of plates	Code
Aug.28, 1978	Lung	Consolidated	2	1, 4
	Liver	Perihepatitis	3	2, 5, 8
	Heart	Pericarditis	3	3, 6, 9
Oct. 4, 1978	Heart	Pericarditis	2	94-1 94-5
	Spleen	?	1	94-3
Feb. 7, 1979	?	?	4	102-1, 102-2, 102-3, 102-4
Sep. 4, 1979	Heart	Pericarditis	2	119-1, 119-3
	Liver	Normal	2	119-2, 119-4
Sep.10, 1979	Yolk-sac	Omphalitis	3	120-1, 120-2, 120-3
		Total	22	

[a]Descriptions were obtained from Bil-Mar Foods Inc.

many as 21 cultures were identified as E. coli by the Entero-Set^{TM}20 identification system (Appendix 1). All cultures from the first, second, and third outbreaks gave the same Entero-Set^{TM}20 results (2331073), while the cultures from subsequent outbreaks yielded different results (Table 3). One culture (120-3) was a gram-positive coccus and was identified as Streptococcus.

O and K Serotypes

The majority of E. coli strains which are pathogenic to poultry belong to only a few serotypes. Therefore, several investigators have screened the E. coli strains that caused fatal infections in poultry using antisera against certain O antigens of E. coli that were most frequently encountered in diseased poultry, e.g. O1, O2, O8, and O78 (Sojka and Carnaghan, 1961; Harry, 1964; Hemsley and Harry, 1965; Heller and Smith, 1973).

In this research, a similar O-antigen screening procedure was applied to the isolates obtained from diseased turkeys. Cultures 9, 94-5, 102-1, and 119-3 were selected for further study, including electron microscopy to determine the presence of pili. Subsequently, screening for K serotypes was done on these cultures. The results are listed in Table 4. Cultures 9 and 102-1 were identified as O78:K80, and cultures 94-5 and 119-3 were identified as O1:K1 and O2:K1, respectively.

Table 3. Entero-Set^{TM}20 numbers of isolates obtained from diseased turkeys

Isolate strain no.[a]	Entero-Set^{TM}20 numbers	Organism
1, 2, 3, 4, 5, 6, 8, 9	2331073	E. coli
94-1, 94-3, 94-5	2331073	E. coli
102-1, 102-2, 102-3, 102-4	2331073	E. coli
119-1	2335073	E. coli
119-2, 119-3	2331033	E. coli
119-4	2233033	E. coli
120-1	2336073	E. coli
120-2	2334073	E. coli
120-3	Gram-positive coccus	Streptococcus

[a]Strain no. corresponds to the code of the plate (Table 2) from which the organism was isolated.

Table 4. O and K titers, and immunodiffusion (ID) of isolates

Cultures	O		K		
	Serum	Titer	Serum	Titer	ID
Controls:					
U5-41 (O1:K1:H7)	O1	1,280	K1	20,480	+
A20a (O2:K1:H6)	O2	640	ND[a]	-	ND
E38 (O78:K80)	O78	1,280	K80	2,560	+
Isolates:					
1, 2, 3, 4 5, 6, 8	O78	1,280	ND	-	ND
9[b]	O78	5,120	K80	5,120	+
94-1	O2	1,280	ND	-	ND
94-3	O1	1,280	ND	-	ND
94-5[b]	O1	1,280	K1	81,920	+
102-1[b]	O78	1,280	K80	40,960	+
102-2, 102-3	O78	1,280	ND	-	ND
102-4	O78	2,560	ND	-	ND
119-1	O?	-	ND	-	ND
119-2	O2	640	ND	-	ND
119-3[b]	O2	640	K1	81,920	+
119-4	O2	320	ND	-	ND
120-1	O?	-	ND	-	ND
120-2	O?	-	ND	-	ND

[a] ND, not done.

[b] Cultures selected for further study.

Electron Microscopy

Electron microscopy of the cells grown on Peptone agar was used to observe pili and flagella on the bacterial cells (Appendices 2, 3 and 4). The average diameter of pili of each culture was calculated and the results are listed in Table 5. The pili of culture 9 had an average diameter of 6.2 nm, while the pili of cultures 94-5 and 119-3 had average diameters of 9.5 and 8.9 nm, respectively.

Hemagglutination Patterns

Two strains possessing mannose-resistant and eluting (MRE) hemagglutinins, H10407 (CFA/I^+) and Moon 263 ($K88ab^+$), were used as controls for hemagglutination (HA). The HA activities of MRE hemagglutinins were better developed when bacteria were grown on CF agar than in CYE broth (Tables 6 and 7). According to Duguid et al. (1955), the optimal condition for the development of MRE hemagglutinins was an overnight incubation on agar at 37°C. The reactions between CFA/I^+ strain and bovine red blood cells (Table 6), and between $K88ab^+$ strain and guinea pig red blood cells (Table 7), developed only at 4°C. All the positive reactions were resistant to D-mannose, but disappeared or were eluted when the bacteria were heated at 65°C for 30 minutes, or when the test mixtures were warmed to 25, 40 and 50°C. These characteristics indicated the presence of a MRE hemagglutinin(s) (Duguid et al., 1979; Evans et al.,

Table 5. Appendages of selected isolates, including the approximate diameter of pili

Isolates	Appendages[a]		Approximate diameter of pili[b] (nm)
	Pili	Flagella	
H10407 (CFA/I$^+$)	+	+	9.0 ± 0.2
9	+	-	6.2 ± 0.1
94-5	+	-	9.5 ± 0.4
102-1	-	-	-
119-3	+	-	8.9 ± 0.5

[a] +, present; -, absent (as determined by electron microscopy).

[b] Means of 10 microscopic observations; $\bar{x} \pm$ SEM (=standard error of means).

Table 6. HA of different species of red blood cells by strain H10407 (CFA/I$^+$) grown at 37^{o}C

Media to grow	Treatment	Temp. of test mixt.	HA with r.b.c. of:[a] Hu	Bv	Ck	Gp	Rm	HA pattern
CFA	Unheated	4^{o}C	R(4+)	R(2+)	R(3+)	N	N	RRRNN
		25^{o}C	R(4+)	N	R(3+)	N	N	
		40^{o}C	N	N	N			
		50^{o}C	N		N			
	Heated[b]	4^{o}C	R(2+)	N	N	N	N	RNNNN
		25^{o}C	N	N	N	N	N	
		40^{o}C	N					
CYE	Unheated	4^{o}C	R(1+)	N	R(2+)	N	N	RNRNN
		25^{o}C	R(1+)	N	R(1+)	N	N	
		40^{o}C	N		N			
		50^{o}C	N		N			
	Heated	4^{o}C	N	N	N	N	N	NNNNN
		25^{o}C	N	N	N	N	N	

[a]Hu, human type A; Bv, bovine; Ck, chicken; Gp, guinea pig; Rm, Rhesus monkey; R, resistant to D-mannose; N, negative HA.

[b]Bacterial suspension was heated at 65^{o}C for 30 minutes.

Table 7. HA of different species of red blood cells by strain Moon 263 (K88ab$^+$) grown at 37°C

Media to grow	Treatment	Temp. of test mixt.	HA with r.b.c. of:[a] Hu	Bv	Ck	Gp	Rm	HA pattern
CFA	Unheated	4°C	N	N	R(3+)	R(2+)	N	NNRRN
		25°C	N	N	R(2+)	N	N	
		40°C			N	N		
		50°C			N			
	Heated[b]	4°C	N	N	N	N	N	NNNNN
		25°C	N	N	N	N	N	
CYE	Unheated	4°C	N	N	R(1+)	S(1+)	S(1+)	NNRSS
		25°C	N	N	N	N	N	
		40°C			N	N	N	
	Heated	4°C	N	N	N	N	N	NNNNN
		25°C	N	N	N	N	N	

[a], [b] See Table 6; S, sensitive to D-mannose.

1979).

The HA patterns using red blood cells of human type A, bovine, chicken, guinea pig, and Rhesus monkey were RRRNN for the CFA/I^+ strain (Table 6) and NNRRN for the K88ab$^+$ strain (Table 7), grown on CFA. The HA pattern of the CFA/I^+ strain was similar to the observation of Evans et al. (1979), which was RRRN without using Rhesus monkey red blood cells. According to Jones and Rutter (1974), the HA activities of K88$^+$ strains with red blood cells of some animal species were not inhibited by D-mannose, but eluted at 37^{o}C.

Heated and unheated cultures 9 and 119-3 exhibited the same HA patterns (NNSSS) when grown on CFA and in CYE broth. Weaker reactions developed when the bacteria were grown in CYE broth (Tables 8 and 9). The HA reactions were eluted or disappeared when the test mixtures were warmed to 25, 40 and 50^{o}C.

The HA pattern of culture 94-5 after growth on CFA was the same as that after growth in CYE broth (Table 10). In both media, the HA pattern developed by unheated culture was SNSSS. After the bacteria were heated at 65^{o}C for 30 minutes, the MS HA with human red blood cells was prevented and the HA pattern was NNSSS. The reactions were eluted when the temperature of test mixtures was raised to 40 and 50^{o}C.

Table 8. HA of different species of red blood cells by culture 9

Media to grow	Treatment	Temp. of test mixt.	HA with r.b.c. of:[a] Hu	Bv	Ck	Gp	Rm	HA pattern
CFA	Unheated	4°C	N	N	S(4+)	S(4+)	S(4+)	NNSSS
		25°C	N	N	S(3+)	S(3+)	S(3+)	
		40°C			S(2+)	S(2+)	S(2+)	
		50°C			S(2+)	S(2+)	S(2+)	
	Heated[b]	4°C	N	N	S(4+)	S(4+)	S(4+)	NNSSS
		25°C	N	N	S(4+)	S(4+)	S(3+)	
		40°C			S(2+)	S(2+)	N	
		50°C			S(2+)	S(2+)	N	
CYE	Unheated	4°C	N	N	S(2+)	S(2+)	S(2+)	NNSSS
		25°C	N	N	S(2+)	S(2+)	S(2+)	
		40°C			S(1+)	S(1+)	S(1+)	
		50°C			S(1+)	S(1+)	S(1+)	
		4°C	N	N	S(2+)	S(2+)	S(2+)	NNSSS
		25°C	N	N	S(2+)	S(2+)	S(2+)	
		40°C			S(1+)	S(1+)	S(1+)	
		50°C			S(1+)	S(1+)	S(1+)	

[a], [b] See Table 6; S, sensitive to D-mannose.

Table 9. HA of different species of red blood cells by culture 119-3

Media to grow	Treatment	Temp. of test mixt.	HA with r.b.c. of:[a] Hu	Bv	Ck	Gp	Rm	HA pattern
CFA	Unheated	4°C	N	N	S(4+)	S(4+)	S(3+)	NNSSS
		25°C	N	N	S(4+)	S(4+)	S(2+)	
		40°C			S(2+)	S(1+)	N	
		50°C			S(2+)	S(1+)	N	
	Heated[b]	4°C	N	N	S(4+)	S(3+)	S(3+)	NNSSS
		25°C	N	N	S(3+)	S(2+)	S(2+)	
		40°C			S(2+)	N	N	
		50°C			S(2+)	N	N	
CYE	Unheated	4°C	N	N	S(3+)	S(3+)	S(3+)	NNSSS
		25°C	N	N	S(3+)	S(3+)	S(3+)	
		40°C			S(2+)	S(2+)	S(1+)	
		50°C			S(2+)	S(1+)	S(1+)	
	Heated	4°C	N	N	S(3+)	S(3+)	S(2+)	NNSSS
		25°C	N	N	S(3+)	S(3+)	S(2+)	
		40°C			S(2+)	S(2+)	S(1+)	
		50°C			S(2+)	S(1+)	S(1+)	

[a], [b] See Table 6; S, sensitive to D-mannose.

Table 10. HA of different species of red blood cells by culture 94-5

Media to grow	Treatment	Temp. of test mixt.	HA with r.b.c. of:[a] Hu	Bv	Ck	Gp	Rm	HA pattern
CFA	Unheated	4°C	S(3+)	N	S(4+)	S(4+)	S(4+)	SNSSS
		25°C	S(2+)	N	S(4+)	S(4+)	S(4+)	
		40°C	N		S(2+)	S(2+)	S(2+)	
		50°C	N		N	N	N	
	Heated[b]	4°C	N	N	S(4+)	S(4+)	S(4+)	NNSSS
		25°C	N	N	S(4+)	S(3+)	S(3+)	
		40°C			S(4+)	S(3+)	S(2+)	
		50°C			S(4+)	S(2+)	S(2+)	
CYE	Unheated	4°C	S(2+)	N	S(3+)	S(4+)	S(3+)	SNSSS
		25°C	S(1+)	N	S(3+)	S(4+)	S(3+)	
		40°C	N		S(2+)	S(3+)	S(1+)	
		50°C	N		S(1+)	S(1+)	N	
	Heated	4°C	N	N	S(4+)	S(4+)	S(4+)	NNSSS
		25°C	N	N	S(3+)	S(4+)	S(4+)	
		40°C			S(2+)	S(2+)	S(2+)	
		50°C			S(1+)	S(1+)	S(1+)	

[a], [b] See Table 6; S, sensitive to D-mannose.

The HA patterns exhibited by culture 102-1 were NNNSS when the bacteria were grown on CFA, NNNSN when the bacteria were grown in CYE broth, and NNNNN when the bacteria were heated after growing in both media (Table 11).

All cultures grown at 18°C, except the controls, exhibited the same HA patterns as those grown at 37°C. No HA reaction was developed by the controls (CFA/I$^+$ and K88ab$^+$ strains) grown at 18°C, which indicated the inhibition of MRE hemagglutinin formation at 18°C. Several investigators have demonstrated that the formation of MRE hemagglutinins such as K88, K99 and CFA was depressed at a growth temperature of 18°C (Ørskov et al., 1975; Burrows et al., 1976; Evans et al., 1978).

In Vitro Adhesion

The control levels for the adhesion tests were determined based on the viable counts of a tissue disc after mixing with the bacterial suspension for two seconds. This method ensured the measurement of a nonspecific carry-over of bacteria on the tissue after washing (Jones and Rutter, 1972). The increase in the viable counts of adhering bacteria between two seconds and certain times of incubation were indicative of mucosal adhesiveness.

Table 11. HA of different species of red blood cells by culture 102-1

Media to grow	Treatment	Temp. of test mixt.	HA with r.b.c. of:[a] Hu	Bv	Ck	Gp	Rm	HA pattern
CFA	Unheated	4°C	N	N	N	S(2+)	S(2+)	NNNSS (NNNNN)
		25°C	N	N	N	N	N	
		40°C				N	N	
	Heated[b]	4°C	N	N	N	N	N	NNNNN
		25°C	N	N	N	N	N	
CYE	Unheated	4°C	N	N	N	S(2+)	N	NNNSN (NNNNN)
		25°C	N	N	N	N	N	
		40°C				N		
	Heated	4°C	N	N	N	N	N	NNNNN
		25°C	N	N	N	N	N	

[a], [b] See Table 6; S, sensitive to D-mannose.

Strain K-12, which possesses type 1 pili, and culture A20a (O2:K1:H6), which was isolated from a human appendix, were used as controls. As shown in Figure 5, D-mannose did not cause a substantial decrease in adhesion. However, the increase in adhesion of an unheated culture of strain K-12 after 50 minutes of incubation was less than one log cycle, therefore, the effect of D-mannose on the adhesion was not clear. Strain K-12 was very sensitive to heat, as indicated by the inability to detect viable cells in the heated culture suspension. However, some of cells appeared to be heat-injured and recovered rapidly during incubation with intestinal tissue. There was a concomitant, rapid increase in the adhesion of viable bacteria to the tissue. The presence of D-mannose appeared to have some effect on the viability of the injured cells. Most of the bacterial cells were destroyed during heating, thus, the adhesion of the heated bacteria was less than that of the unheated bacteria, even after 50 minutes of incubation.

The increase in the adhesion of heated and unheated cells of culture A20a was also less than one log cycle (Figure 6). However, culture A20a was more heat resistant than strain K-12 as indicated by the relatively high counts of the heated culture suspension (1.0×10^7 cells/ml).

In the absence of D-mannose, heated and unheated cultures 9 and 119-3 showed an increase of about 1.25 to

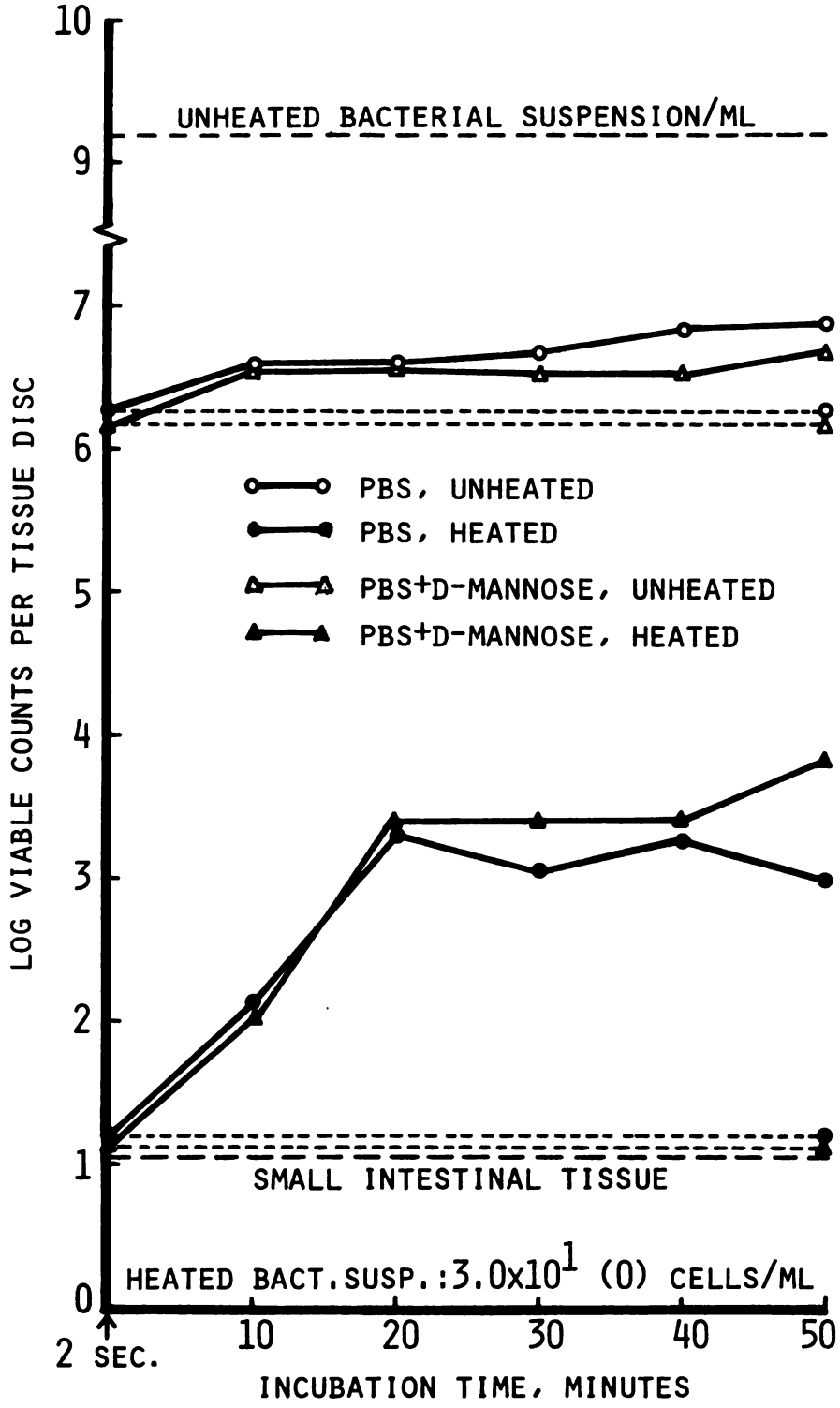

FIGURE 5. ADHESION OF STRAIN K-12 TO TURKEY SMALL INTESTINAL TISSUE.

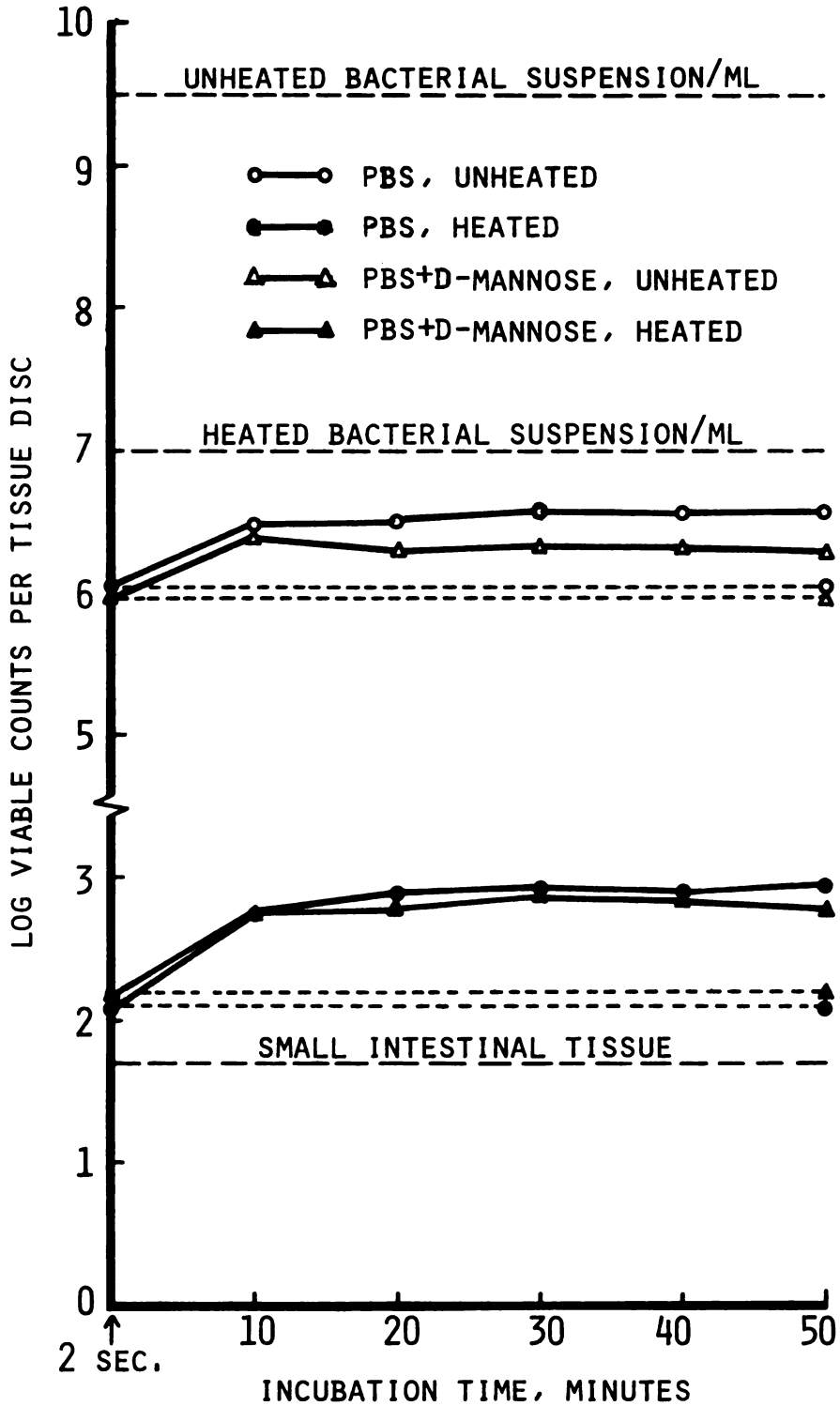

FIGURE 6. ADHESION OF CULTURE A20a (O2:K1:H6) ISOLATED FROM HUMAN TO TURKEY SMALL INTESTINAL TISSUE.

1.75 log cycles in the viable bacteria adhered on tissue after 50 minutes of incubation (Figures 7 and 8). However, in the presence of 0.5 percent D-mannose, the increase in adhesion after 50 minutes of incubation was less than tenfold; the greatest increase occurred during the first 20 minutes of incubation. There were large decreases in the viable counts of the bacterial suspension and tissue containing bacteria after heating at 65°C for 30 minutes. After 50 minutes of incubation in the absence of D-mannose the log increases in the adhesion of heated bacteria were close to those of the unheated bacteria. When cultures 9 and 119-3 were grown at 18°C (Figures 9 and 10), fewer bacteria adhered to the tissue than when the bacteria had been grown at 37°C. During incubation there was virtually no increase in the adhesion of heated cells of cultures 9 and 119-3 grown at 18°C, except for culture 9 in the presence of D-mannose.

In the presence or absence of 0.5 percent D-mannose, there was a tenfold increase in unheated cells of culture 94-5 adhered to the tissue after 50 minutes of incubation (Figure 11). However, in the absence of D-mannose, the adhesion of cells increased during the entire incubation period, while maximum adhesion occurred after 20 minutes of incubation in the presence of D-mannose. In the absence of D-mannose, cells of culture 94-5 grown at 18°C (Figure 12) had a lower rate of adhesion than those grown

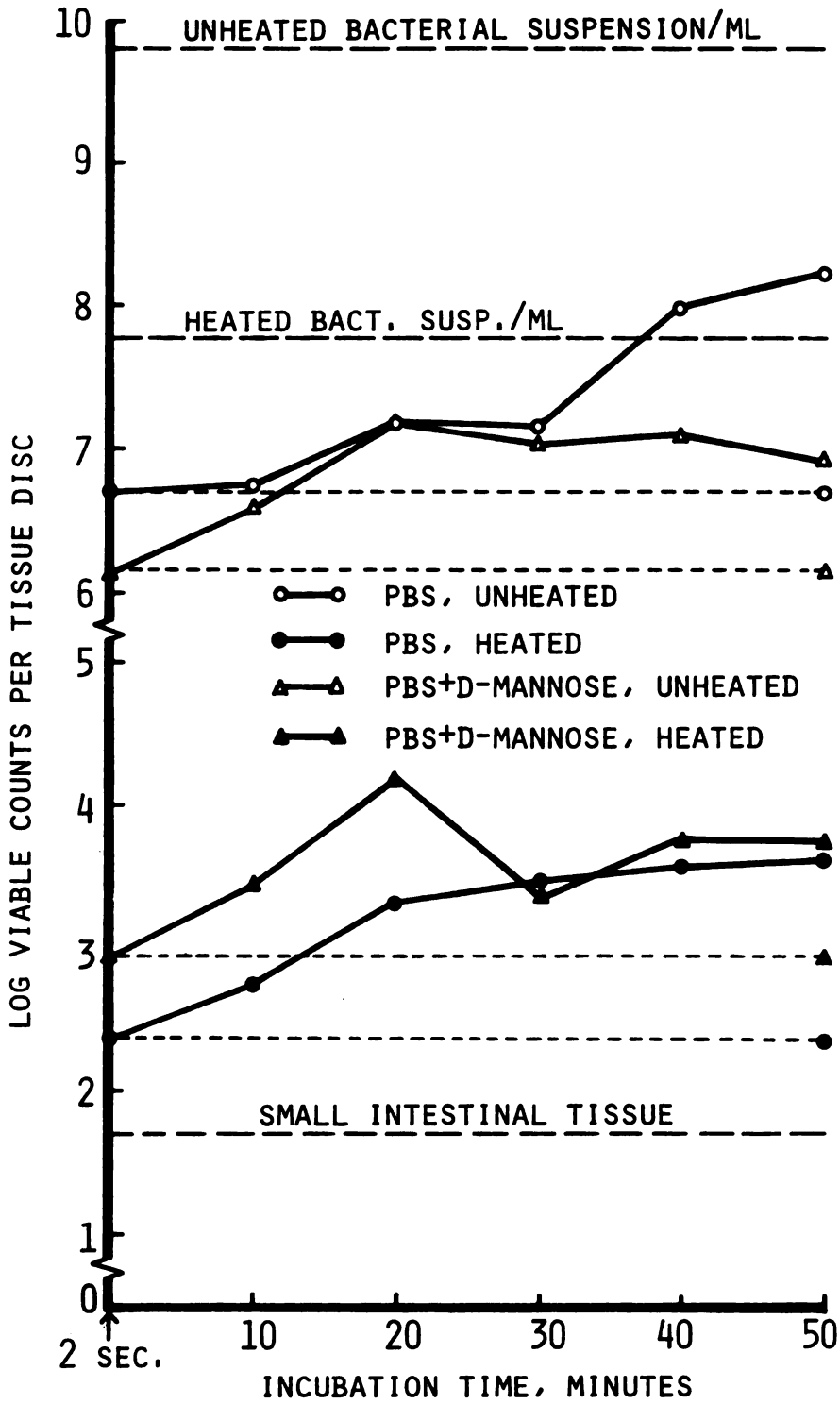

FIGURE 7. ADHESION OF CULTURE 9, GROWN AT 37°C, TO TURKEY SMALL INTESTINAL TISSUE.

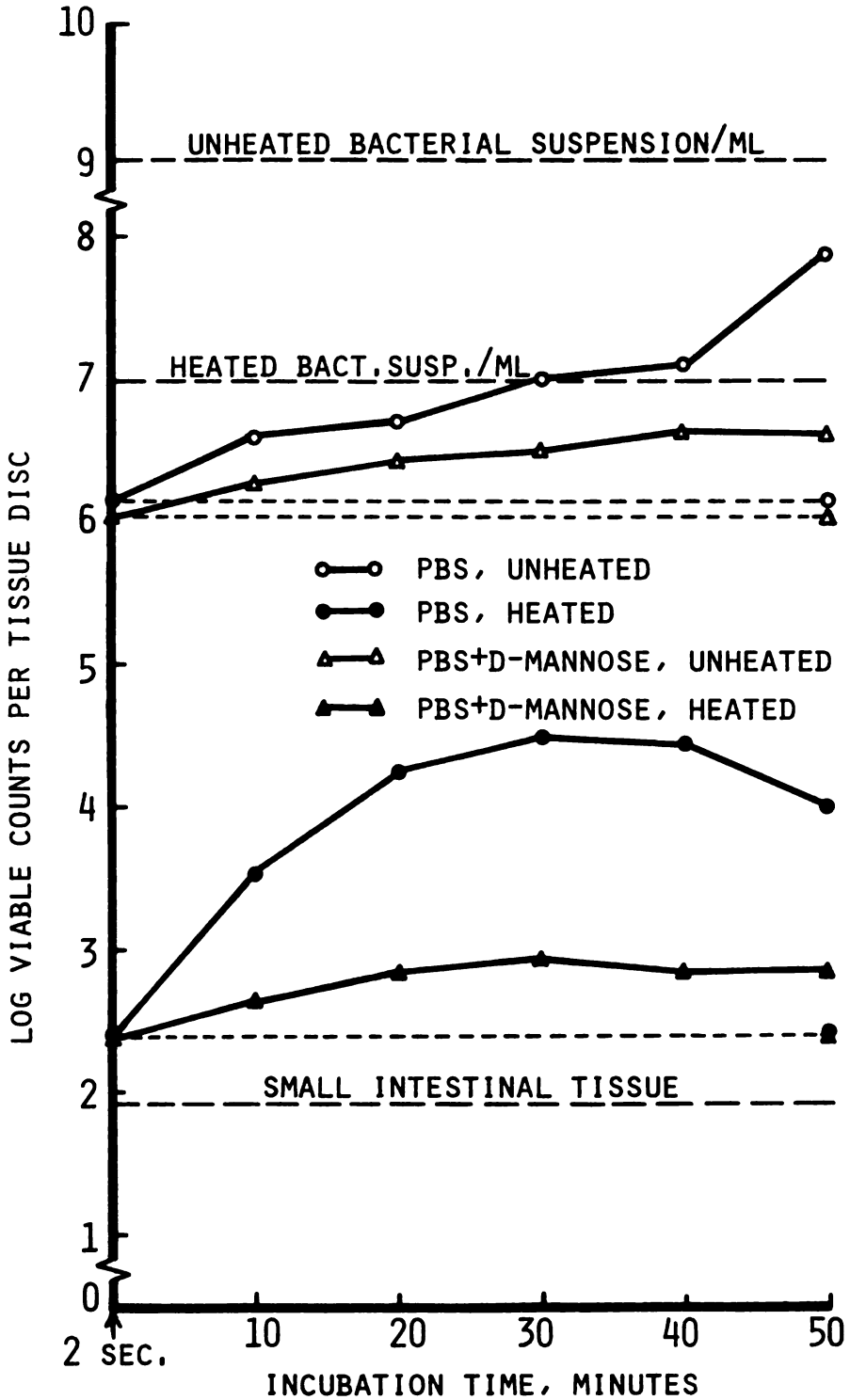

FIGURE 8. ADHESION OF CULTURE 119-3, GROWN AT 37°C, TO TURKEY SMALL INTESTINAL TISSUE.

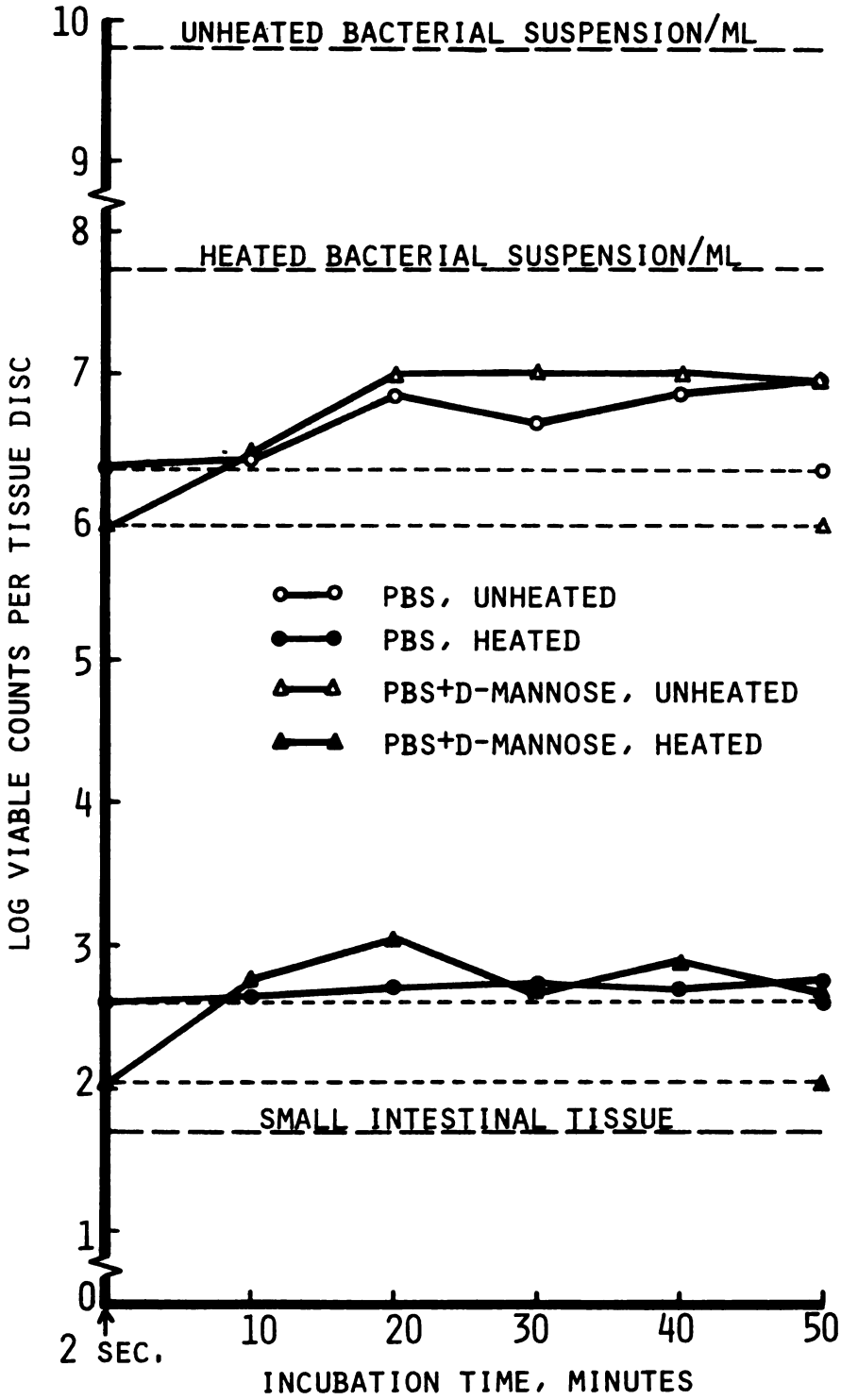

FIGURE 9. ADHESION OF CULTURE 9, GROWN AT 18°C, TO TURKEY SMALL INTESTINAL TISSUE.

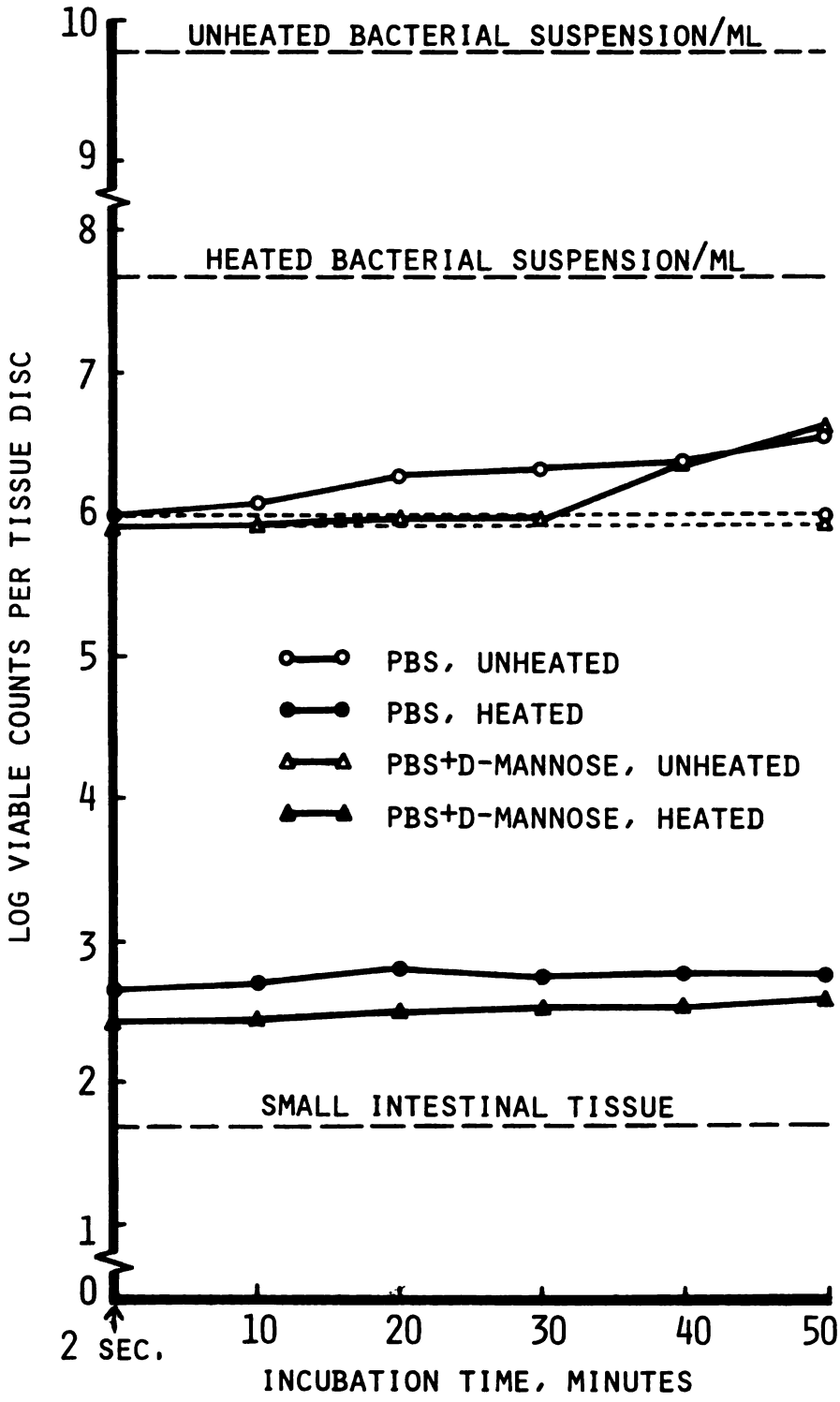

FIGURE 10. ADHESION OF CULTURE 119-3, GROWN AT 18°C, TO TURKEY SMALL INTESTINAL TISSUE.

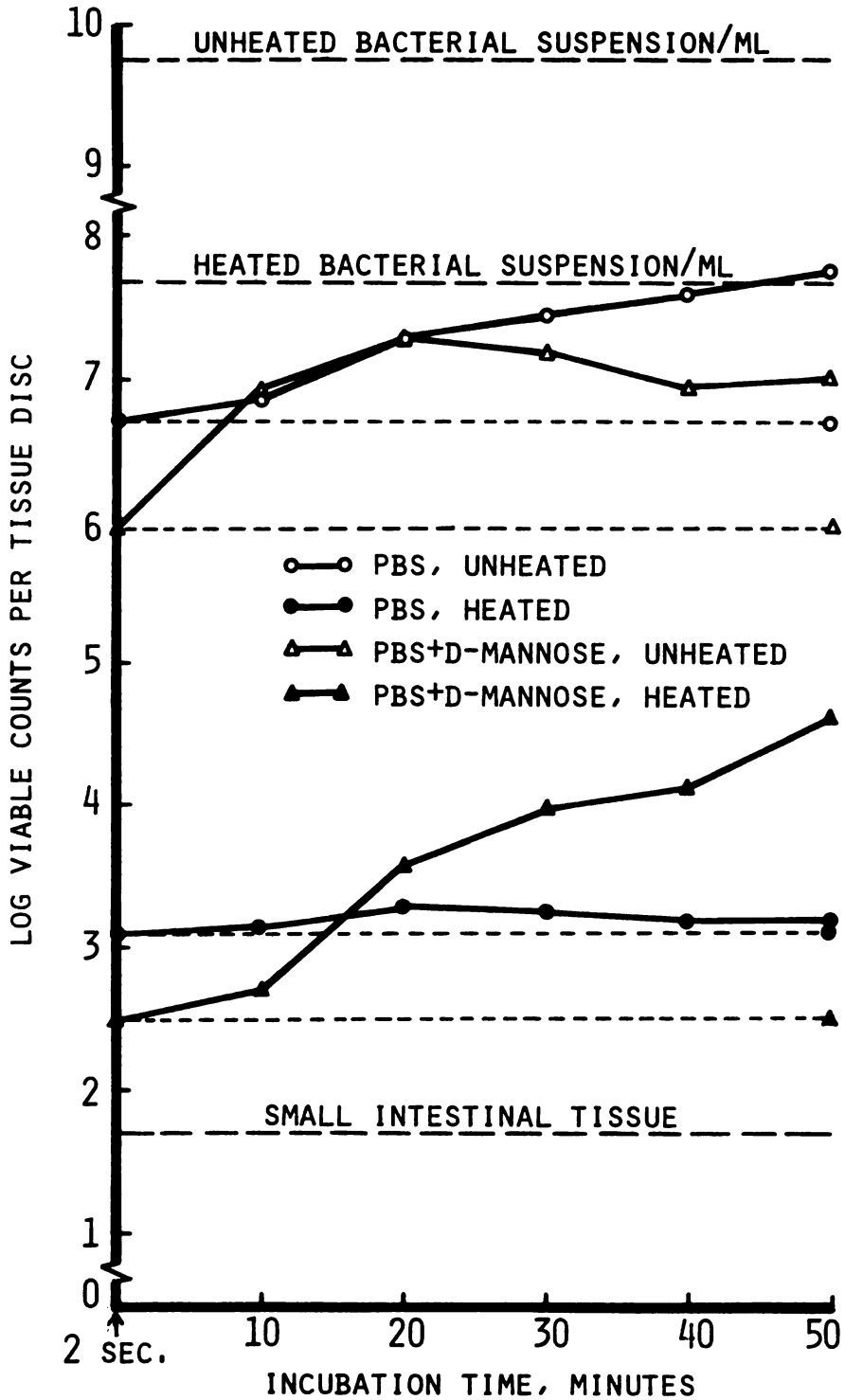

FIGURE 11. ADHESION OF CULTURE 94-5, GROWN AT 37°C, TO TURKEY SMALL INTESTINAL TISSUE.

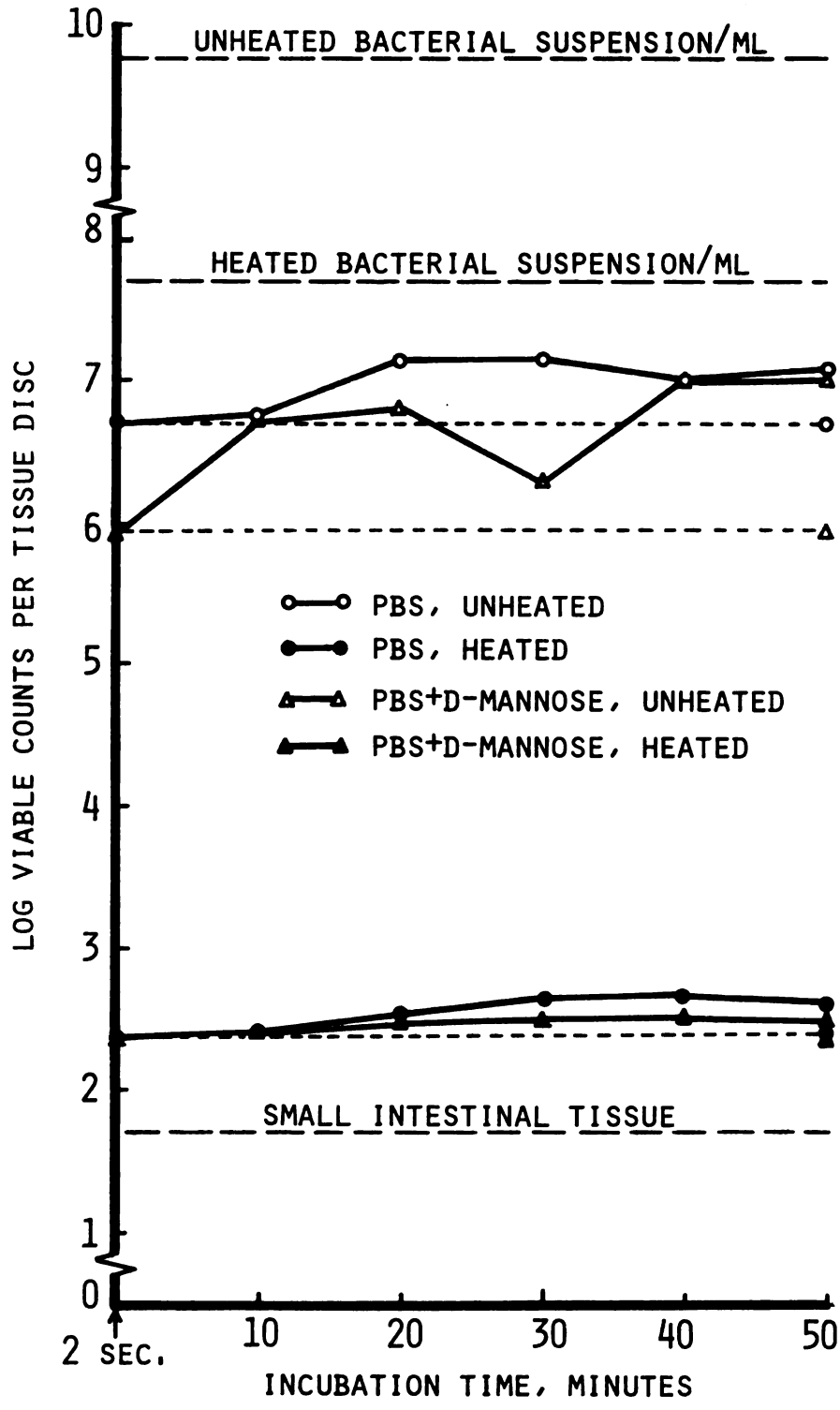

FIGURE 12. ADHESION OF CULTURE 94-5, GROWN AT 18°C, TO TURKEY SMALL INTESTINAL TISSUE.

at 37°C. This result is similar to those reported for cultures 9 and 119-3.

Culture 102-1 had weak or negative hemagglutination reactions with animal red blood cells (Table 11). To some extent the unheated cells adhered to the epithelial tissue of turkey small intestine (Figures 13 and 14), although the increase in adhesion after 50 minutes of incubation was much lower than that of the other cultures.

Inhibition of In Vitro Adhesion

Inhibition by Sugars and Sugar Derivatives

Culture 119-3 was used to study the effects of various sugars and sugar derivatives on the adhesion of E. coli to turkey small intestinal tissue. This culture had the highest degree of adhesion among all the cultures tested, and was identified as O2:K1, a serotype commonly found in fatal infections in poultry. All sugars and sugar derivatives, except D-glucose at 0.5 percent and N-acetylneuraminic acid at 0.5 and 1.0 percent, caused a statistically significant, but partial inhibition ($P<0.01$) of E. coli adhesion to turkey small intestinal tissue (Figures 15 and 16). However, only inhibition by D-mannose and α-methyl-D-mannoside at 0.5 and 1.0 percent concentrations resulted in more than one log cycle decrease in the adhesion. Adhesion was not inhibited completely even by 1.0 percent D-mannose or α-methyl-D-mannoside.

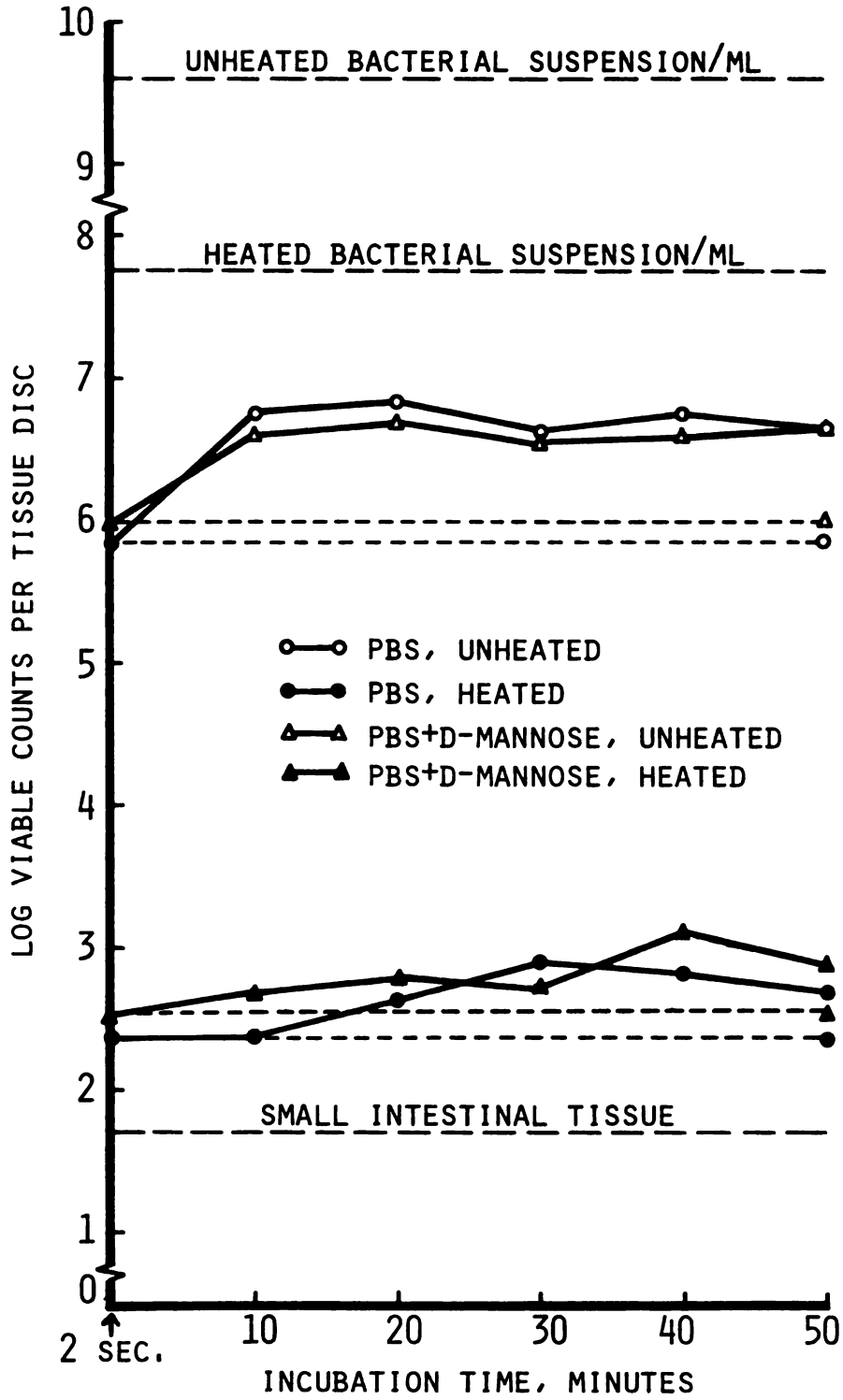

FIGURE 13. ADHESION OF CULTURE 102-1, GROWN AT 37°C, TO TURKEY SMALL INTESTINAL TISSUE.

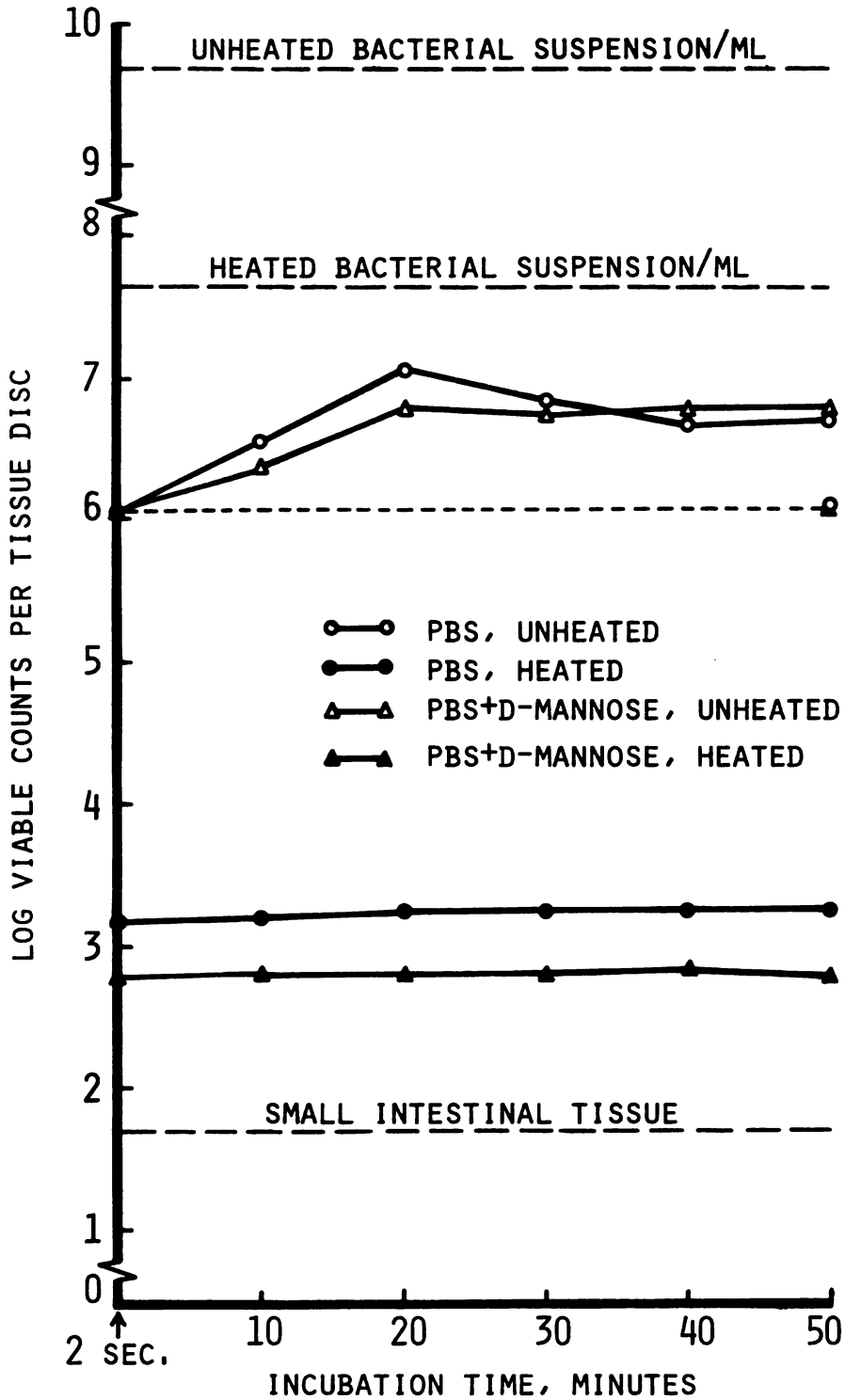

FIGURE 14. ADHESION OF CULTURE 102-1, GROWN AT 18°C, TO TURKEY SMALL INTESTINAL TISSUE.

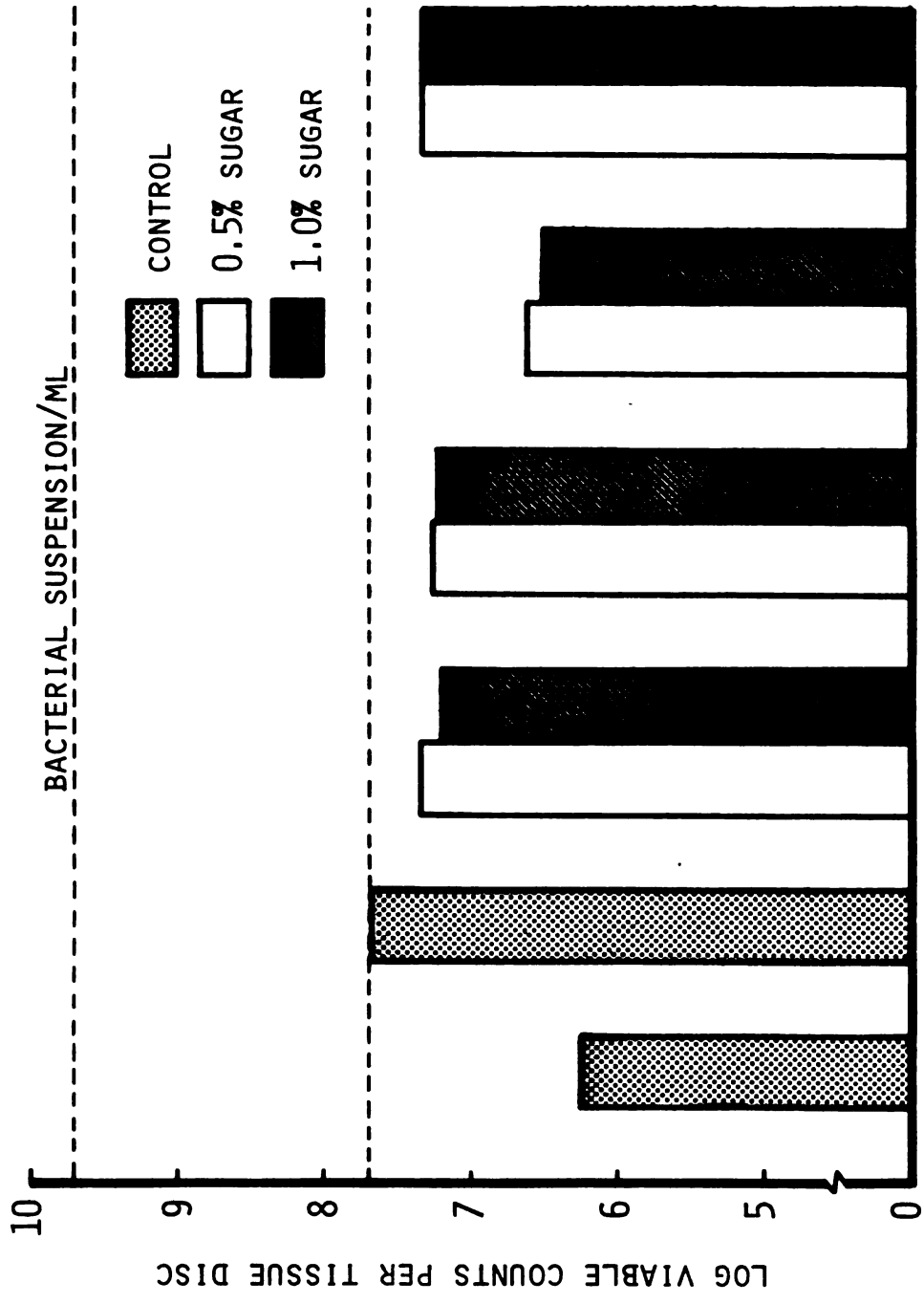

FIGURE 15. INHIBITION OF E. coli (CULTURE 119-3) ADHESION TO TURKEY SMALL INTESTINAL TISSUE BY SUGARS AFTER 50 MINUTES OF INCUBATION.

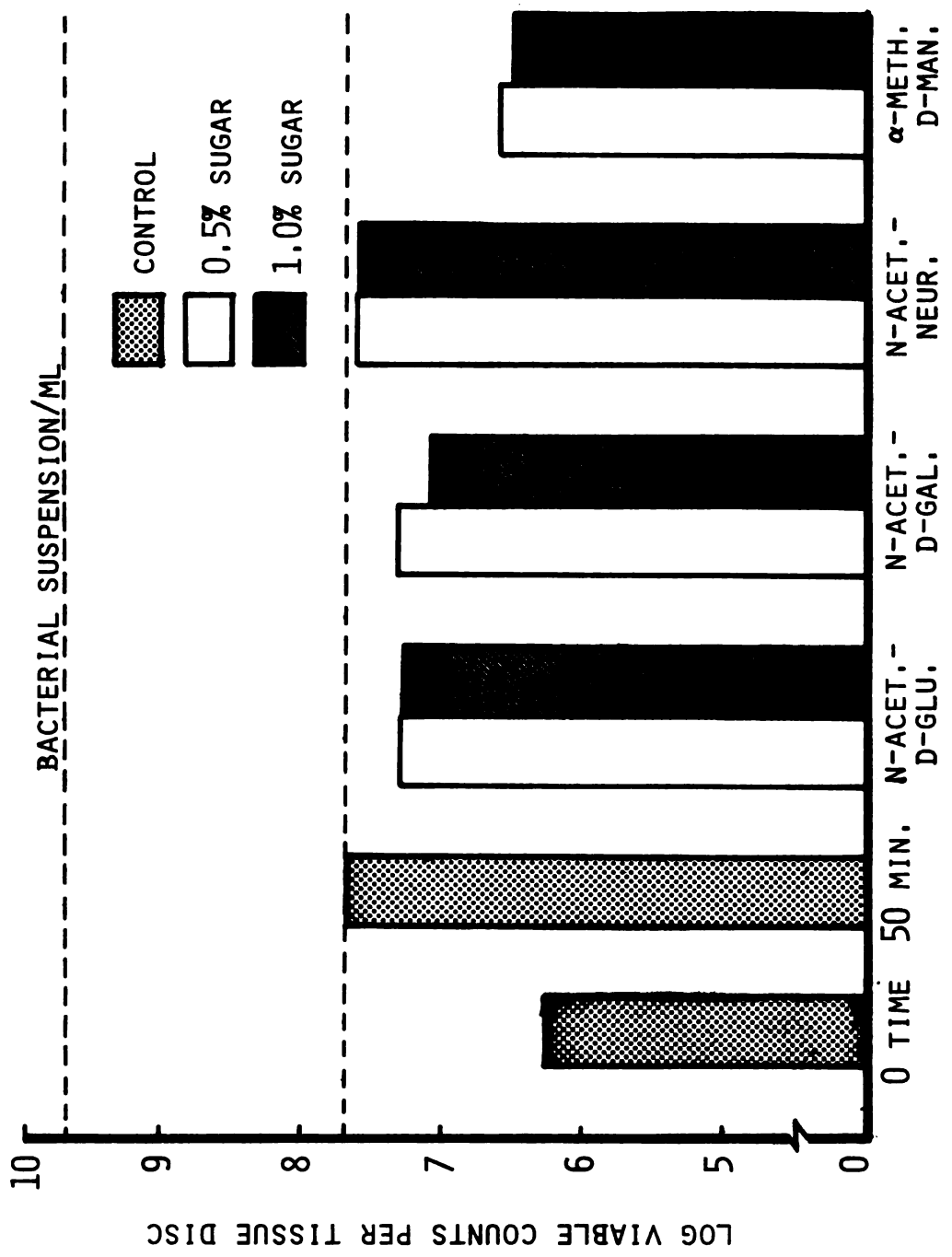

FIGURE 16. INHIBITION OF E. coli (CULTURE 119-3) ADHESION TO TURKEY SMALL INTESTINAL TISSUE BY SUGAR DERIVATIVES AFTER 50 MINUTES OF INCUBATION.

Figure 17 shows that the minimum concentration of D-mannose and α-methyl-D-mannoside which caused a detectable inhibition in the adhesion of E. coli (culture 119-3) to turkey small intestinal tissue was 0.1 percent. A large increase in inhibition occurred when concentrations were increased to 0.4 percent for D-mannose and 0.5 percent for α-methyl-D-mannoside. Although additional inhibition occurred at concentrations above 0.5 percent, adhesion was not completely inhibited up to a concentration of 2.5 percent sugars.

Inhibition by Concanavalin A and Sodium Metaperiodate

Figure 18 shows that concanavalin A greatly inhibited the adhesion of E. coli (culture 119-3) to turkey small intestinal tissue. Inhibition occurred almost linearly up to a concentration of 0.20 percent (w/v), and above this concentration, no further detectable inhibition occurred. At a minimum concentration of 0.10 percent (w/v), sodium metaperiodate completely inhibited the adhesion of E. coli to turkey small intestinal tissue.

Inhibition by Isolated Pili

When isolated pili were present at a concentration of 2.0 mg protein per ml there was approximately one log cycle decrease in the adhesion of E. coli (culture 119-3) to turkey small intestinal tissue (Figure 19). At higher

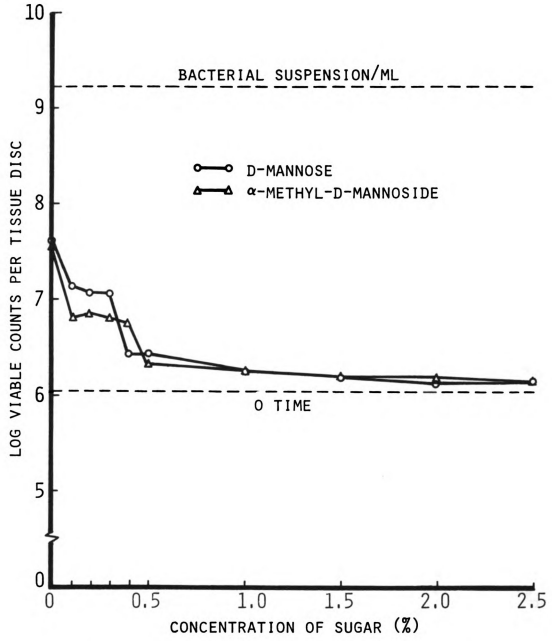

FIGURE 17. INHIBITION OF **E. coli** (CULTURE 119-3) ADHESION TO TURKEY SMALL INTESTINAL TISSUE BY D-MANNOSE AND α-METHYL-D-MANNOSIDE AFTER 50 MINUTES OF INCUBATION.

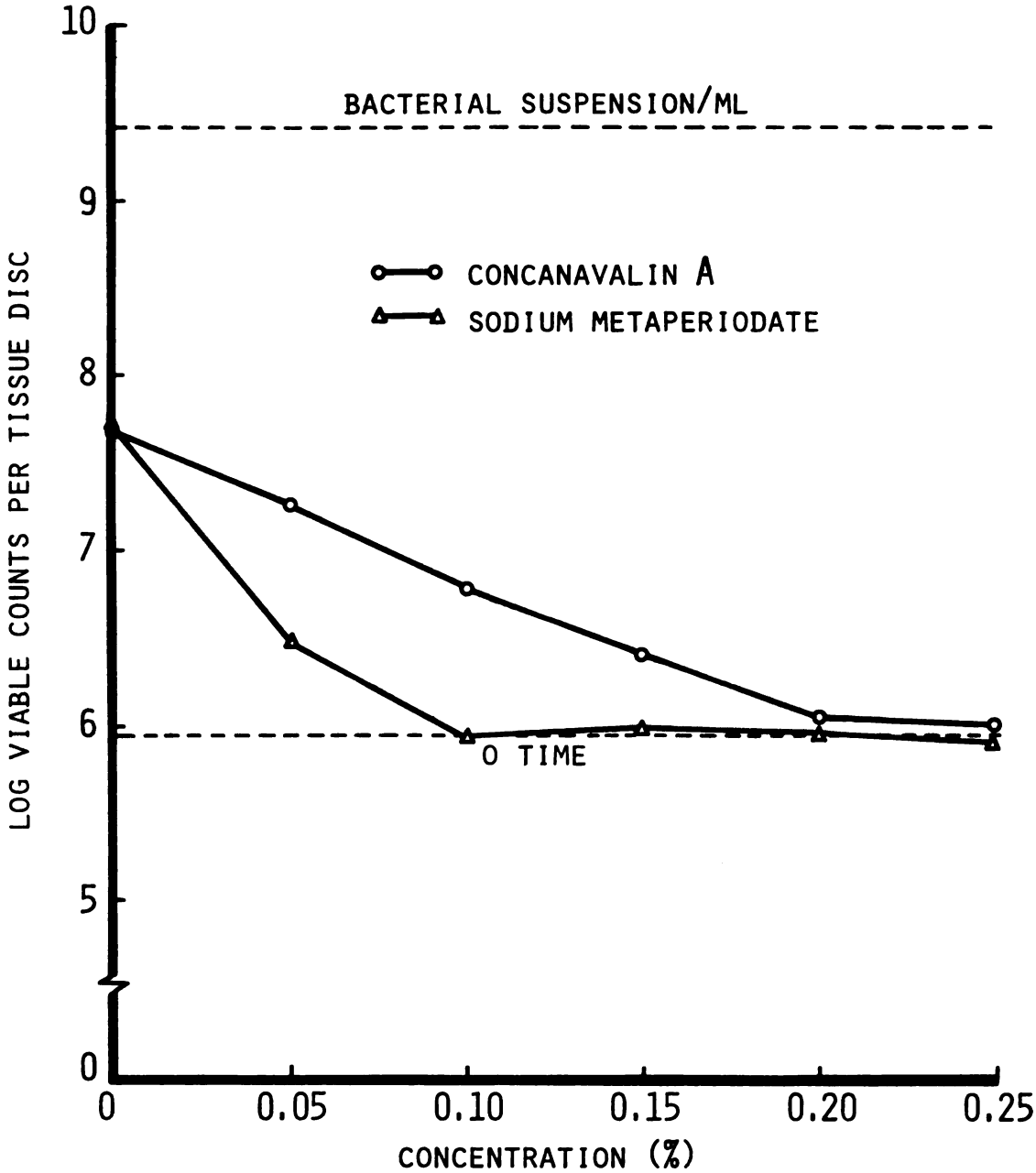

FIGURE 18. INHIBITION OF **E. coli** (CULTURE 119-3) ADHESION TO TURKEY SMALL INTESTINAL TISSUE BY CONCANAVALIN A AND SODIUM METAPERIODATE AFTER 50 MINUTES OF INCUBATION.

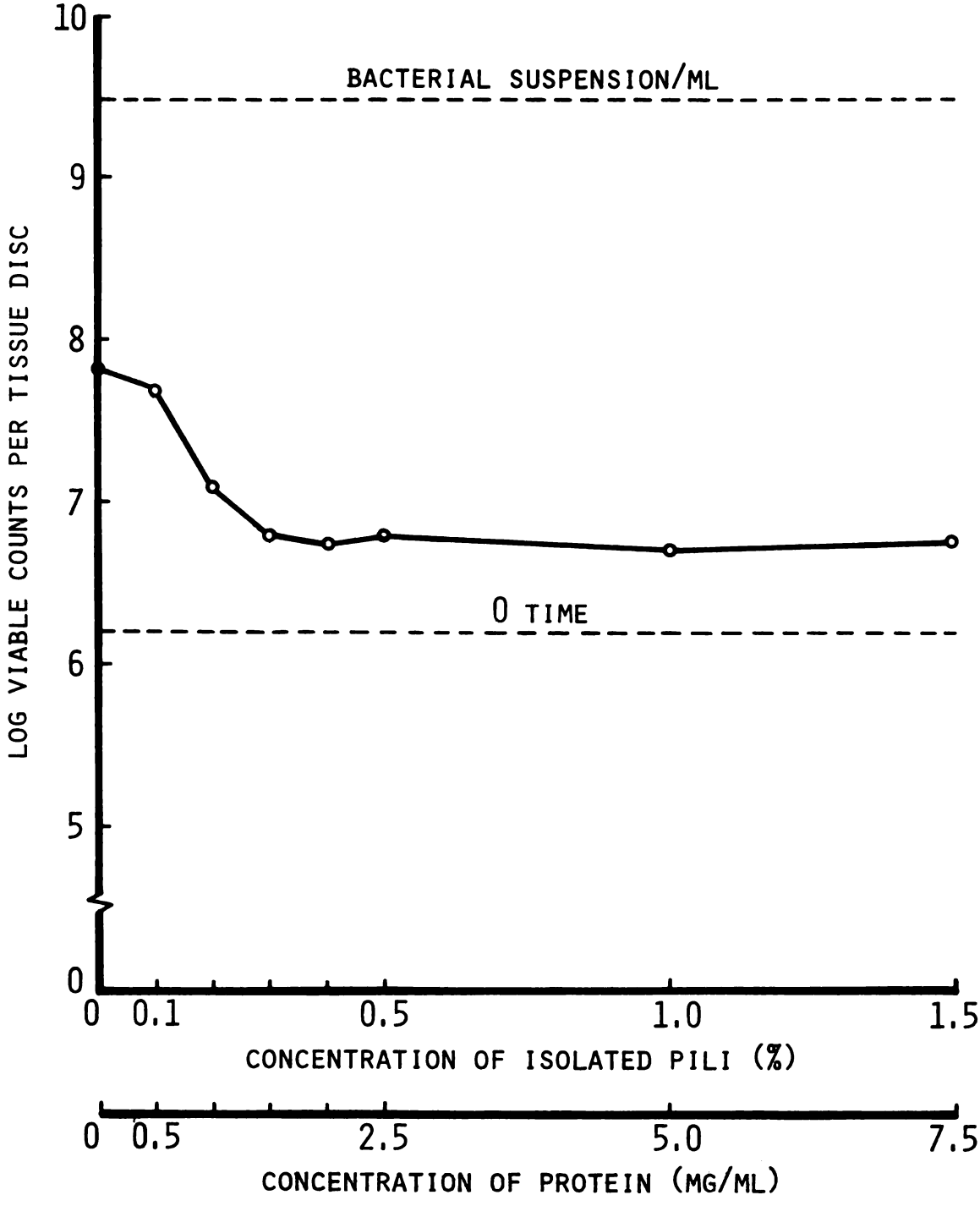

FIGURE 19. INHIBITION OF **E. coli** (CULTURE 119-3) ADHESION TO TURKEY SMALL INTESTINAL TISSUE BY ISOLATED PILI AFTER 50 MINUTES OF INCUBATION.

concentrations of the isolated pili, no further decrease in adhesion was detected, and the adhesion was not completely inhibited.

Isolated Pili

Chromatography of the ammonium sulfate precipitate of culture 119-3 on a Sephadex G-50 column yielded a single, large peak (Figure 20). Fractions 30 to 50 were pooled and lyophilized. After SDS gel electrophoresis, only one band was observed in gels prepared using the $(NH_4)_2SO_4$ precipitate and the pooled fractions (Figure 21), indicating the presence of only one protein before and after chromatography. Using the standard curve the protein from isolated pili had a molecular weight of about 34,800 (Figure 22).

Protein concentrations of the lyophilized precipitate and pooled fractions, determined by the Lowry method, were 49 and 51 percent, respectively, indicating the presence of nonprotein impurities in the lyophilized samples. Since the purpose of this study was to study the effect of pili on adhesion, no attempt was made to purify the protein or to determine its specific hemagglutination activity. The precipitate and the pooled fractions were not dialyzed before determining their protein concentrations. However, several previous studies have shown that pili consisted of almost 100 percent protein (Brinton and Stone, 1961; Stirm et al., 1967; Isaacson, 1977). Preparations of the pili, before and after chromatography, are shown in Figure 23.

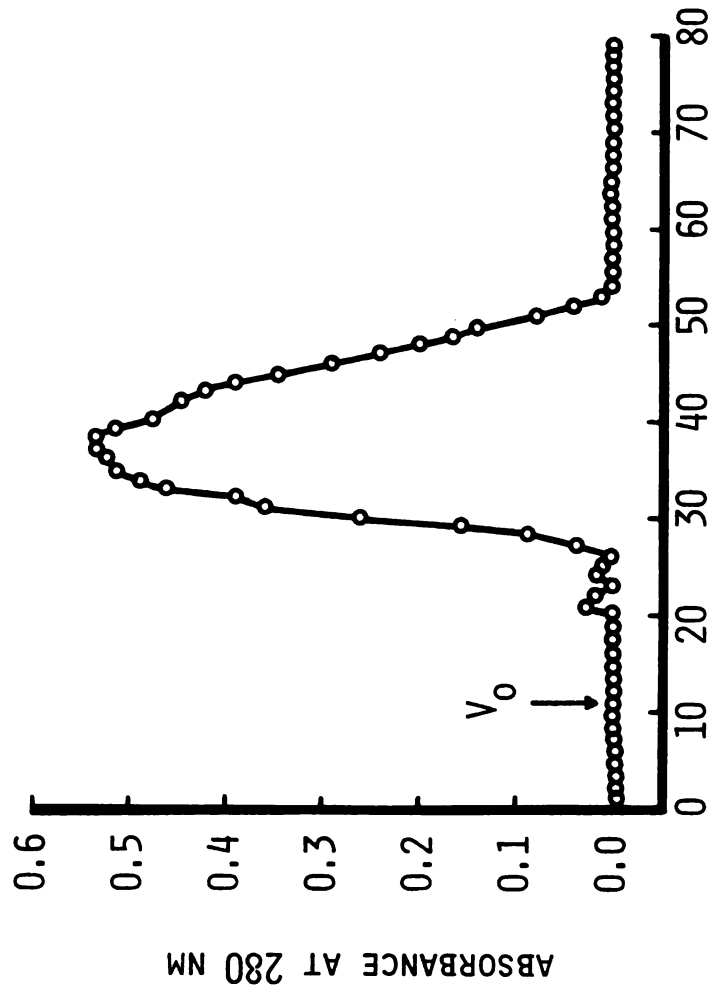

FIGURE 20. ELUTION PATTERN OBTAINED FROM THE CHROMATOGRAPHY OF AMMONIUM SULFATE PRECIPITATE (CULTURE 119-3 PILI) ON A SEPHADEX G-50 COLUMN.

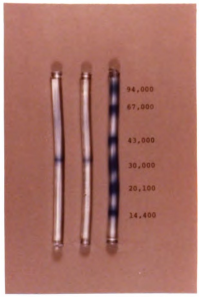

Figure 21. SDS-gels of (A) Ammonium sulfate precipitate of pili; (B) Pili after chromatography on a Sephadex G-50 column; (C) Standard proteins.

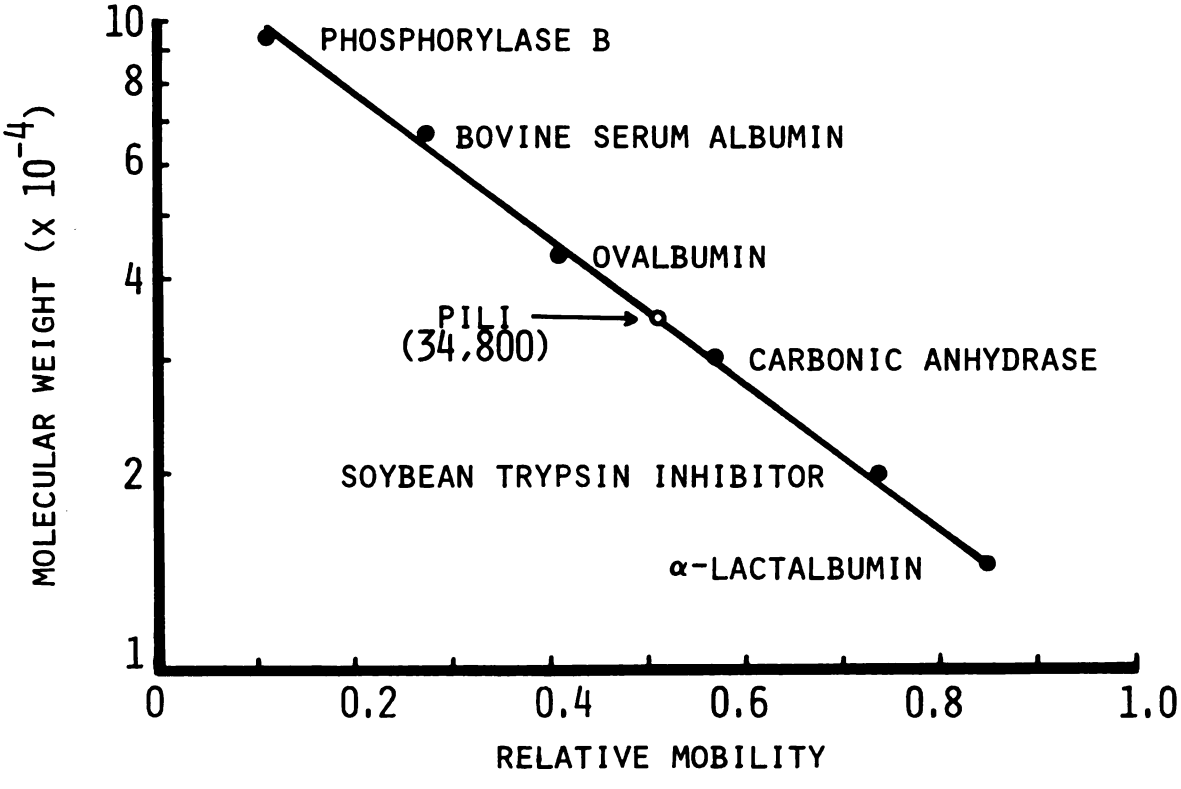

FIGURE 22. STANDARD CURVE FOR THE DETERMINATION OF APPROXIMATE MOLECULAR WEIGHT OF CULTURE 119-3 PILI BY SDS-GEL ELECTROPHORESIS.

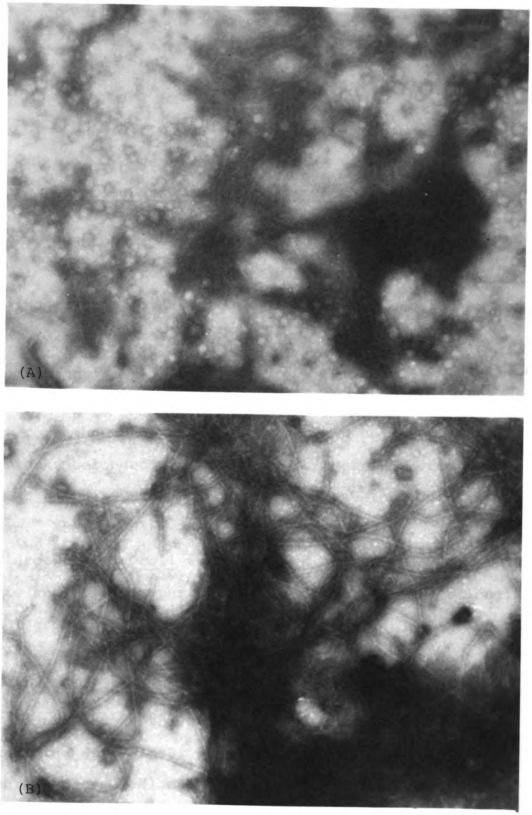

Figure 23. Electron micrographs of (A) Ammonium sulfate precipitate of culture 119-3 pili; (B) Pili after chromatography on a Sephadex G-50 column; Concentration, 10 mg/ml; Magnification, x 64,000.

DISCUSSION

E. coli Serotypes Found in Diseased Poultry

Sojka and Carnaghan (1961), and Heller and Smith (1973) have reported that over 60 percent of the E. coli strains causing fatal infections in chickens belonged to O1:K1, O2:K1, and O78:K80 serotypes. Sojka and Carnaghan (1961) also isolated strains in the O8 group from cases of coli-septicemia in chickens. However, Harry (1964) found that more than half of the strains from coli-septicemia outbreaks belonged to the O2 group, about 25 percent belonged to the O78 group, and 8 percent belonged to the O1 group. According to Hemsley and Harry (1965), O1, O2 and O78 groups of E. coli were involved in outbreaks of omphalitis and coliform pericarditis, with 65 percent belonging to O2 group. The same serotypes were detected in E. coli cultures isolated from diseased turkeys in Michigan; they were identified in the O1, O2 and O78 groups. No culture was identified in the O8 group. Cultures 9 and 102-1 were identified as O78:K80, and cultures 94-5 and 119-3 were identified as O1:K1 and O2:K1, respectively. Cultures 9, 94-5, and 119-3 were isolated from the hearts of turkeys with pericarditis. The screening procedure for serotyping

of E. coli was a rapid method for the presumptive identification of O and K serotypes of E. coli isolated from diseased poultry, however, it might not give a complete identification of O and K serotypes due to the possibility of cross-reactions between O antigens of E. coli, and the selected O and K antisera used.

Size of E. coli Pili

Several investigators have reported pili from E. coli of varying diameter, including type 1 pili which had a diameter of 7 nm (Brinton et al., 1964), K99 with a diameter between 7.0 and 9.8 nm (Isaacson, 1977), and pili from other E. coli strains which had a diameter between 5 and 7 nm (Korhonen et al., 1980). Electron photomicrographs from this investigation show that culture 9 had the smallest pili, with an average diameter of 6.2 nm, while culture 94-5 had the largest pili with an average diameter of 9.5 nm. The length of the pili was not measured due to the irregular length of the pili.

Hemagglutination Activities of E. coli

The HA pattern (NNSSS) exhibited by cultures 9 and 119-3 indicated the presence of a mannose-sensitive (MS) hemagglutinin, and was one of the HA type III patterns (NNSS without Rhesus monkey red blood cells) described by Evans et al. (1979). However, the HA reactions were eluted or disappeared when the test mixtures were warmed to 40 and

50°C, which disagreed with one of the characteristics of MS hemagglutinins described by Duguid et al. (1979). Of the 170 HA type III cultures tested by Evans et al. (1979), 82 percent belonged to EPEC serogroups, 5 percent were CFA-negative ETEC, 2 percent were Shigella-like serogroups, and 8 percent were isolated from children with diarrhea but did not belong to the known EPEC serogroups. The majority of the HA type III strains which did not belong to the above serogroups have been reported to be associated with sporadic diarrhea and extraintestinal infections in humans (Czirok et al., 1976, 1977; Ørskov et al., 1977; Evans et al., 1979), including some strains which were cross-reactive with Salmonella and EPEC serogroups (Evans et al., 1979) The HA type III phenotype might be responsible for the virulence of E. coli. Evans et al. (1979) suggested that EPEC might possess a specific virulence factor known as MS hemagglutinin. No individual somatic antigen or serogroup has been reported to relate consistently to the HA type III phenotype, and the surface factors responsible for this HA pattern have not been determined. The HA type III pattern could be related to the possession of type 1 pili (SNSS; HA type IV), and another factor which masked MS HA of human red blood cells so that a NNSS (type III) pattern was observed rather than a SNSS (type IV) pattern. Figure 24 shows a schematic diagram representing the interrelationship among ETEC, EPEC, Shigella-like strains and non-EPEC. Region

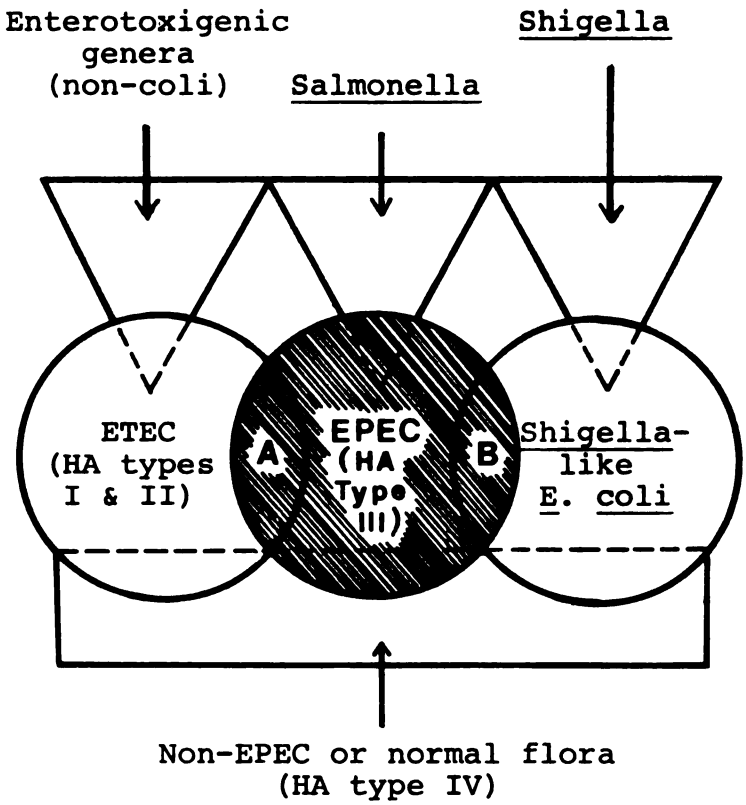

Figure 24. Schematic diagram representing interrelationship between ETEC, EPEC, Shigella-like E. coli, and non-EPEC (Evans et al., 1979).

A represents the ETEC isolates which belong to EPEC serogroups and those ETEC which exhibit the HA type III phenotype. Region B represents the Shigella-like isolates which exhibit the HA type III phenotype. Region C represents the facultatively enteropathogenic E. coli (FEEC) groups which are recognized as normal flora but exhibit type III phenotype and may cause sporadic diarrhea, enteritis, and extraintestinal infections.

Culture 9 was identified as O78:K80, whereas culture 119-3 was identified as O2:K1. O2 serotypes were found as normal flora in humans and were not recognized as EPEC (Ørskov et al., 1977). In addition, Evans et al. (1979) found that O2 strains were associated with sporadic diarrhea and extraintestinal infections. O2 and O78 serotypes were two of the 18 O-serotypes proposed by Evans et al. (1979) as FEEC. These two O-serotypes have also been reported as strains which commonly cause fatal infections in poultry, including coli-septicemia, omphalitis, and pericarditis (Sojka and Carnaghan, 1961; Harry, 1964; Hemsley and Harry, 1965; Heller and Smith, 1973). Evans et al. (1979) suggested that non-EPEC serogroups could be considered as enteropathogens on the basis of at least two of the following criteria: (1) possession of HA type III phenotype, (2) cross-reaction with Salmonella serogroups, (3) cross-reaction with EPEC serogroups, (4) association with sporadic diarrhea or enteritis, with or without septicemia,

and (5) association with extraintestinal infections. The presence of a certain serogroup in stools or intestine is not a primary indication that the culture is an EPEC since these E. coli serogroups are known to be members of the normal flora. Initiation of disease by EPEC, according to Sakazaki et al. (1974), was dependent on the special ability of the bacteria to multiply in the intestine of the host and thereby develop a large population. Although cultures 9 and 119-3 possessed HA type III phenotype and associated with extraintestinal infections (pericarditis), these cultures should not be considered as human enteropathogens based on the criteria given by Evans et al. (1979) since these cultures were isolated from turkeys.

The HA pattern (SNSSS) exhibited by culture 94-5 was similar to the HA type IV phenotype, and it was exhibited by many strains including strain K-12 which was known to possess type 1 pili (Evans et al., 1979). Of the 74 cultures tested by Evans et al. (1979) and known to possess this HA type IV phenotype, 31 percent belonged to EPEC serogroups, 12 percent were CFA-negative ETEC, and 16 percent were isolated from children with diarrhea but did not belong to the known EPEC serogroups.

The HA pattern of heated culture 94-5 was NNSSS, similar to the pattern of the HA type III. Thus, this culture may possess only type 1 pili. However, the negative HA of the heated culture with human red blood cells

and the eluted reactions when the temperature of test mixtures was raised to 40 and 50°C were unexpected (Duguid et al., 1979). Another possible explanation of the result is that culture 94-5 possessed two MS hemagglutinins, a type III hemagglutinin which was heat resistant and responsible for MS HA with chicken, guinea pig, and Rhesus monkey red blood cells, and another responsible for MS HA of human red blood cells and possibly chicken, guinea pig and Rhesus monkey red blood cells, but its HA activity was destroyed when the culture was heated at 65°C for 30 minutes. Evans et al. (1979) suggested that at least two factors, the type 1 pili and an unidentified factor, were associated with MS HA of EPEC. MS, as well as MRE, hemagglutinins of E. coli might function in adhesion of bacteria to host cells (Salit and Gotschlich, 1977; Evans et al., 1979).

The HA patterns NNNSS and NNNSN (or NNNS without Rhesus monkey red blood cells) exhibited by unheated culture 102-1 after growth on CFA and in CYE broth, respectively, were not included in the HA patterns described by Evans et al. (1979). The reactions were very weak (2+) in all MS HA. These results could be expected since electron microscopy of culture 102-1 after growth on Peptone agar revealed no pili were present.

Adhesive Properties of E. coli

Jones and Rutter (1972) reported an increase of 1.6 to 2.0 log cycle in the viable counts of piglet intestinal tissue incubated for 30 minutes with a K88-positive E. coli, and less than a tenfold increase in the viable counts when the tissue was incubated with a K88-negative strain. In this research, cultures 9 and 119-3 showed an increase of about 1.25 to 1.75 log cycles in the viable bacteria adhered on turkey small intestinal tissue after 50 minutes of incubation. D-mannose caused partial inhibition in the adhesion of cultures 9 and 119-3 to turkey small intestinal tissue. This result could be indicative of the fact that more than one carbohydrate-containing receptor for E. coli was present in the intestinal epithelial cells, or that the concentration of D-mannose was not high enough to cause complete inhibition. Another possibility is that there was nonspecific adhesion of bacteria, which was not mediated by pili, in the presence of D-mannose. The hemagglutination reactions of these cultures with animal red blood cells were completely inhibited by D-mannose at a concentration of 0.5 percent, indicating that mannose-containing receptors were the only receptors or were adjacent to the receptors in the red blood cells for the bacterial pili. Culture A20a, which was isolated from human appendix and had the same O:K serotype (O2:K1) as culture 119-3, adhered to turkey small intestinal tissue at much lower

rate than culture 119-3, indicating that the host and/or tissue specificities influenced the adhesion of E. coli to animal cells, as was suggested by Smith (1972, 1976).

Schaeffer et al. (1979) considered two different attachment mechanisms occurred in the adhesion of bacteria to human or animal epithelial cells, one was the temporary binding which happened as soon as bacteria were in contact with the epithelial cells, and the second was a more stable form of adhesion. The stable adhesion was related to the limited number of receptors on the epithelial cells for the bacterial pili (Jones et al., 1976; Isaacson et al., 1978; Schaeffer et al., 1979). Accordingly, in the absence of D-mannose, the adhesion of heated and unheated cultures 9 and 119-3 during the first 20-30 minutes of incubation would be due to the temporary binding. A more stable adhesion occurred upon prolonged incubation. However, in the absence of D-mannose, some of the bacteria, such as the heated culture 119-3, could not develop a more stable adhesion, and bacteria were readily detached from the epithelial cells. In the presence of D-mannose, the competition between the receptors and D-mannose for the bacterial pili also resulted in a decrease in the amount of bacteria adhered to the tissue. Jones et al. (1976) considered such a reduction in the number of bacteria attached to epithelial cells to be associated with the spontaneous loss or denaturation of the adhesive component (adhesin) on the

bacterial surface.

The thermal death times of E. coli cells generally are between 20 and 30 minutes at 57.3°C (Frazier and Westhoff, 1978). Thus, a large portion of cultures 9 and 119-3 were killed or possibly injured during heating at 65°C for 30 minutes, and would not be detected even if they had adhered to the epithelial cells. The HA of these cultures were resistant to heating at 65°C for 30 minutes. After 50 minutes of incubation in the absence of D-mannose, the increases in the adhesion of heated bacteria were close to those of the unheated bacteria, indicating the repair of some of the injured cells. Ray and Speck (1973ab) reported that repair of E. coli cells injured during freezing occurred in foods during one hour at 25°C; most of the injured cells could grow on a non-selective medium such as Trypticase soy agar containing 0.3 percent yeast extract (TSYA), but not in selective media such as Violet red bile agar (VRBA) or Deoxycholate-lactose agar (DLA). A similar condition might occur with the heat-injured bacteria incubated with the intestinal tissue, however, there might be a difference in the injuries and repair mechanisms between heat-injured cells and freezing-injured cells.

Partial inhibition in the pili formation might have occurred when cultures 9 and 119-3 were grown at 18°C since fewer bacteria adhered to the tissue than when the bacteria had been grown at 37°C. However, growth at 18°C did not

affect the hemagglutination reactions. Interestingly, upon prolonged incubation, the viable counts of bacteria adhering to the tissue increased. The increase in the amount of adhesion might be due to reformation of pili when the culture was brought to a higher temperature, i.e. incubation at room temperature during the adhesion tests. The presence of D-mannose appeared to affect the attachment, since larger increases in the adhesion of bacteria occurred in the presence of D-mannose. Novotny et al. (1969) studied the reformation of E. coli pili after the pili were removed by blending; they reported that the length of type 1 pili increased from 35 to 90 percent of the control length in 30 minutes at 37°C. Cultures 9 and 119-3 were more sensitive to heat when grown at 18°C, and no apparent repair of the cells occurred even during incubation with the tissue. Frazier and Westhoff (1978) stated E. coli was more heat-resistant when grown at 38.5°C than at 28°C.

Culture 94-5 was more heat sensitive than cultures 9 and 119-3 as indicated by the relatively low numbers of heated bacteria adhering to the tissue. However, the presence of D-mannose seemed to have some effect(s) on the repair and/or adhesion of this culture since the apparent adhesion of heated bacteria increased in the presence of D-mannose. When culture 94-5 had been grown at 18°C, the presence of D-mannose also might have some effect(s) on the reformation of pili during incubation at room temperature.

The absence of adhesion of cells of this culture heated at 65°C for 30 minutes was also indicative of their increased sensitivity to heat when the culture was grown at 18°C for 72 hours.

Electron microscopy showed the absence of pili in culture 102-1, therefore, it was concluded that the increase in the adhesion of this culture to turkey small intestinal tissue, which was much lower than that of the other cultures, was due to nonspecific adhesion, i.e. was not mediated by pili. This suggestion was supported by the fact that the presence of D-mannose did not affect the adhesion of cells grown at 37 and 18°C, indicating a mannose-containing receptor was not involved in the adhesion of these bacteria.

Inhibition of In Vitro Adhesion

Recent reports (Jones and Freter, 1976; Ofek et al., 1977, 1978; Salit and Gotschlich, 1977) indicate that some bacteria may bind to carbohydrate molecules as receptors on epithelial cell surfaces. Thus, if specific carbohydrates are added to the incubation mixture, adhesion is inhibited due to competition between carbohydrates and carbohydrate-containing receptors for the adhesion, in this case, pili. These studies have shown that hemagglutination, caused by adhesion of cultures 9, 94-5 and 119-3 to animal red blood cells, was completely inhibited by

D-mannose. D-mannose and α-methyl-D-mannoside resulted in more than one log cycle decrease in the adhesion of E. coli (culture 119-3) to turkey small intestinal tissue. The results suggested that saturation of binding sites on the bacterial surface, in this case pili, by D-mannose and α-methyl-D-mannoside prevented the adhesion of the bacteria to the epithelial membrane receptors, which presumably contained mannose or a mannose-like structure. The inhibition of adhesion by D-mannose and α-methyl-D-mannoside is similar to the results reported by Ofek et al. (1977, 1978) who suggested that the binding of E. coli to human epithelial cells was mediated by a mannose-specific, lectin-like substance present in the pili which bound to mannose or mannose-like receptor sites on the mucosal cells. However, adhesion of culture 119-3 to turkey small intestinal tissue was not completely inhibited up to a concentration of 2.5 percent sugars. The incomplete inhibition probably was due to the reversible binding of D-mannose and α-methyl-D-mannoside to the pili, so that some bacteria were able to replace the sugars and bind to the epithelial cells.

The inhibition of adhesion by other sugars, which resulted in less than one log cycle decrease in the adhesion, might be due to a less powerful binding of the sugars or sugar derivatives to the bacterial surface. Jones (1975) suggested that the β-D-galactosyl residue of the mucosal

glycoprotein in the pig intestine might be involved in the adhesion of E. coli, but the adhesion could not be inhibited by a simple sugar or by a small oligosaccharide. In this experiment, N-acetyl-D-galactosamine at 1.0 percent concentration caused a partial (0.6 log cycle) decrease in the adhesion of E. coli to turkey small intestinal tissue.

Concanavalin A and sodium metaperiodate greatly inhibited the adhesion of E. coli (culture 119-3) to turkey small intestinal tissue. Concanavalin A is a lectin that binds to D-mannose and D-glucose residues (Sharon and Lis, 1972), while sodium metaperiodate is an oxidative compound known to cleave the C-C bond between the vicinal hydroxyl groups of sugars. The inhibition of adhesion caused by these compounds lends credence to the hypothesis that mannose or mannose-like receptors are important in the adhesion of E. coli to turkey small intestinal tissue.

Since the in vitro adhesion of E. coli to intestinal epithelial cells has been suggested to be mediated by pili (Jones and Rutter, 1972; Isaacson et al., 1977, 1978; Moon et al., 1977; Nagy et al., 1977), isolated pili should act as competitive inhibitors of bacterial adhesion. The presence of isolated pili decreased the adhesion of E. coli (culture 119-3) to turkey small intestinal tissue, however, the adhesion was not completely inhibited. The incomplete inhibition of adhesion by isolated pili might be due to a decrease in the adhesive activity of the isolated pili as

compared to the native, attached pili. Isaacson et al. (1978) reported that purified homologous pili at a concentration of 2000 ug per ml successfully competed for the adhesion of E. coli strains possessing 987P or type 1 pili on pig intestinal epithelial cells; however, addition of purified K99 had a variable effect on the adhesion of $K99^+$ strain, including either competition or enhancement of adhesion. These investigators suggested that some specific effects, which were variable from experiment to experiment, resulted from the addition of purified, homologous pili.

CONCLUSIONS

The E. coli cultures, isolated from diseased turkeys during several disease outbreaks in Michigan, consisted primarily of three O-serogroups, O1, O2 and O78 groups. Culture 9 and 102-1 were identified as O78:K80, while cultures 94-5 and 119-3 were identified as O1:K1 and O2:K1, respectively. These bacteria possessed pili with a diameter between 6.2 and 9.5 nm.

The cultures isolated from diseased turkeys exhibited mannose-sensitive hemagglutination activities (MS HA), indicating that they possessed MS hemagglutinins. Cultures 9 and 119-3 exhibited one of the type III HA patterns (NNSSS), which has been found in some human EPEC strains. Culture 94-5 exhibited one of the type IV HA patterns (SNSSS), which also has been found in some human EPEC strains and in E. coli strains that possessed type 1 pili.

Although HA activities of the culture isolated from diseased turkeys were substantially affected by heating at 65^{o}C for 30 minutes, the in vitro adhesive properties were greatly affected by heating. Heating at 65^{o}C for 30 minutes caused reductions of more than two log cycles in the numbers of bacteria adhering to turkey small intestinal

tissue. Growth at 18°C rather than 37°C also resulted in reductions in adhesion, although growth at that temperature did not affect the HA activities. During incubation at room temperature the numbers of bacteria adhering to turkey small intestinal tissue increased by ca. 1.25 to 1.75 log cycles for cultures 9 and 119-3, and by ca. 1.0 log cycle for culture 94-5.

Among the sugars tested, only D-mannose and α-methyl-D-mannoside had a marked inhibitory effect on the adhesion of culture 119-3 to turkey small intestinal tissue. D-mannose and α-methyl-D-mannoside at concentrations of 0.4 and 0.5 percent, respectively, reduced the numbers of bacteria adhering to the turkey small intestinal tissue by 1.0 log cycle; however, adhesion was not completely inhibited at concentrations of these compounds as high as 2.5 percent. Concanavalin A and sodium metaperiodate at concentrations of 0.2 and 0.1 percent, respectively, completely inhibited the adhesion of culture 119-3 to turkey small intestinal tissue. Adhesion was substantially inhibited by isolated pili, however, at concentrations higher than 2.0 mg protein per ml, no further inhibition was detected. The isolated pili were less inhibitory than D-mannose, α-methyl-D-mannoside, concanavalin A, and sodium metaperiodate. SDS gel electrophoresis indicated protein from the isolated pili had a molecular weight of ca. 34,800.

Further research should be directed towards the study of *in vivo* adhesion of the cultures isolated from diseased turkeys to turkey small intestinal tissue. Studies of the effects of different sugars, sugar derivatives, and isolated pili on the *in vivo* adhesion and other factors which affect adhesion could yield information which may be used to reduce the incidence and/or severity of *E. coli* infections in poultry.

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APPENDIX

Appendix 1

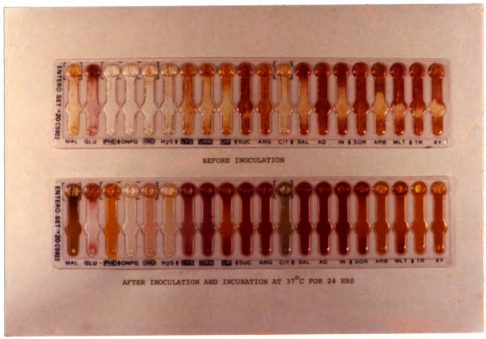

Figure A1. Entero-Set^{TM}20 identification system, using culture 119-3.

Appendix 2

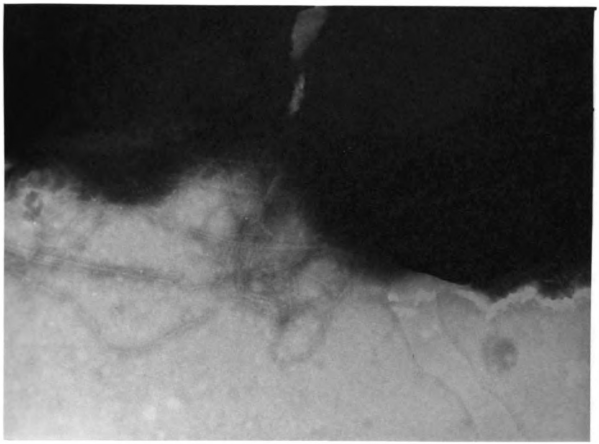

Figure A2. Electron micrograph of negatively stained cells of E. coli (culture 9) grown on Peptone agar at 37°C; Magnification, x 103,000.

Appendix 3

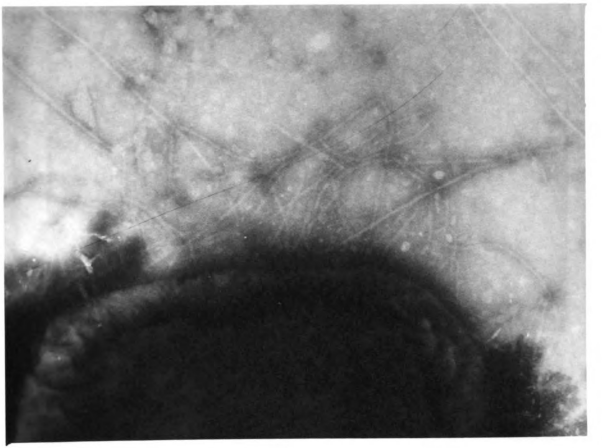

Figure A3. Electron micrograph of negatively stained cells of E. coli (culture 94-5) grown on Peptone agar at 37°C; Magnification, x 103,000.

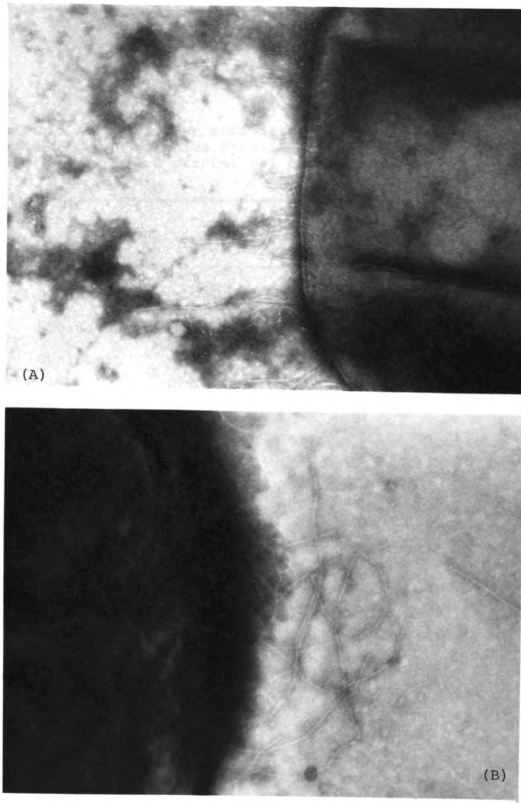

Figure A4. Electron micrographs of negatively stained cells of E. coli (culture 119-3) grown on Peptone agar at 37°C; Magnification, (A) x 60,000, (B) x 103,000.

Appendix 5

Table A1. Relative mobilities of standard proteins and pilus protein on SDS-gels at 7.5 and 10.0 percent gel concentrations

Proteins	Relative mobility	
	7.5% gel	10.0% gel
Phosphorylase b	0.11	0.05
Bovine serum albumin	0.28	0.10
Ovalbumin	0.41	0.20
Carbonic anhydrase	0.56	0.40
Soybean trypsin inhibitor	0.74	0.63
α-lactalbumin	0.85	0.74
Pilus protein	0.52	0.36