A Rapid Bioluminescence Method for Quantifying Bacterial Adhesion to Polystyrene

By M. J. HARBER,* RUTH MACKENZIE AND A. W. ASSCHER

Department of Renal Medicine, Welsh National School of Medicine, K.R.U.F. Institute, Royal Infirmary, Cardiff CF2 1SZ, U.K.

(Received 30 March 1982; revised 5 July 1982)

Bioluminescence ATP analysis has been used to assess bacterial adhesion with hydrophobic polystyrene tubes as the attachment surface. The assay was performed at 37 °C and pH 6.8 with a 10 min incubation period. A variation of more than 200-fold was observed in the adherence capacity of 34 urinary isolates of *Escherichia coli*, and organisms could be classified as strongly or weakly adherent. All strains capable of strong adhesion possessed both type 1 fimbriae and flagella, and maximum adhesion was expressed during the exponential growth phase. Attachment was in all cases virtually eliminated by addition of 2.5% (w/v) D-mannose to the incubation buffer. Conversely, strains which were deficient in type 1 fimbriae or flagella, or both, were weakly adherent during all phases of growth. There was no correlation between adherence of *E. coli* to polystyrene and adherence to buccal or uroepithelial cells, but there was a significant association with adherence to uromucoid ($P < 0.002$).

INTRODUCTION

It is widely believed that the adherence of bacteria to mucosal surfaces is an important first stage in the pathogenesis of many infectious diseases. A variety of biological systems have been used to study and measure bacterial adherence *in vitro*, most methods using exfoliated or freshly isolated epithelial cells as the attachment surface. There is some uncertainty, however, whether sloughed-off cells are truly representative of intact epithelia, while a high level of colonization of epithelial cells with indigenous organisms can often be a practical problem. Furthermore, the presence of uromucoid (Tamm-Horsfall protein) in preparations of uroepithelial cells can present a serious source of error since bacterial adherence to human uroepithelial cells is mediated predominantly by mannose-resistant (MR) fimbriae, while adhesion to mucus (which may coat the cell surface) is associated with mannose-sensitive (MS) type 1 fimbriae (Ørskov *et al.*, 1980a; Chick *et al.*, 1981).

Recent studies have shown that fresh urinary bacterial isolates are non-fimbriate and non-adherent to buccal and uroepithelial cells (Harber *et al.*, 1982a), results which indicate that fimbrial-mediated adhesion is not a virulence factor for bacteria within the lumen of the urinary tract. However, these observations do not preclude a role for adhesion in tissue invasion within the renal parenchyma which can occur once the pathogens have passed through the urinary tract (Harber *et al.*, 1982b). Some MR fimbriae recognize as a receptor for attachment a carbohydrate component of the P blood group antigens which are commonly found on the surface of epithelial cells (Källénus *et al.*, 1980; Leffler & Svanborg Edén, 1980). These adhesins, which have been designated P fimbriae, appear to be a marker of virulence for *Escherichia coli* in early episodes of symptomatic pyelonephritis (Väisänen *et al.*, 1981; Källénus *et al.*, 1981).

MS fimbriae appear to play no part in the attachment of uropathogens to human uroepithelial cells (Ørskov *et al.*, 1980a; Chick *et al.*, 1981), but an important distinction must be made

Abbreviations: MR, mannose-resistant; MS, mannose-sensitive.

between humans and animal models in this respect since several reports have demonstrated mannose-sensitive bacterial adherence to rat bladder cells (Aronson *et al.*, 1979) and rodent bladders *in vivo* (Fader *et al.*, 1979; Chick & Asscher, 1980). There are, in addition, two aspects in which bacterial adherence mediated by MS fimbriae may be of clinical significance. Firstly, mannose-sensitive adherence may be an important factor in the host-parasite relationship within tissues as MS fimbriae facilitate attachment of bacteria to professional phagocytes in the absence of antibody and complement (Silverblatt *et al.*, 1979; Mangan & Snyder, 1979; Bar-Shavit *et al.*, 1980). Secondly, the ability of uromucoid to trap MS fimbriate bacteria may represent an important host defence mechanism within the urinary tract (Ørskov *et al.*, 1980*b*), and since this adherence cannot be measured directly, due to the impracticability of quantifying urinary mucus, an indirect method of study is desirable.

Because of the variable nature of biological surfaces some workers have used solid surfaces of glass or polystyrene to study bacterial attachment since these materials provide more stable and better defined surfaces. Purified type 1 fimbriae adhere by their tips to polystyrene latex particles (Brinton, 1965), and hence polystyrene would appear to be a highly suitable material for quantifying bacterial adhesion mediated by type 1 fimbriae, and for studying the surface properties of the bacterial cell which are relevant to the attachment process. The adherence of organisms to glass and polystyrene has been quantified by staining washed preparations and relating the absorbance of the stain to bacterial numbers (Fletcher, 1976; Rutter & Abbott, 1978), while methods for measuring bacterial adherence to epithelial cells rely on the use of radio-labelled cultures or on direct microscopic examination (Chick *et al.*, 1981). A more simple and potentially more accurate and sensitive method for determining cell numbers is to measure extractable ATP using the firefly bioluminescence system (Harber, 1982). Indeed, this technique has already been used successfully to determine viable cell mass in dental plaque (Robrish *et al.*, 1978; Distler *et al.*, 1980).

In this communication we present a simple and rapid bioluminescence method for measuring bacterial adherence using hydrophobic polystyrene tubes as the attachment surface. The method is used to assess the properties of the bacterial cell which influence attachment of a series of urinary isolates of *E. coli* to polystyrene, and to compare the ability of these organisms to adhere to polystyrene with their ability to adhere to isolated epithelial cells and uromucoid.

METHODS

Organisms. A total of 34 urinary isolates of *E. coli* were selected for study. Strains 7, 10, 12, 20, 23, 44, 49, 56, 57, 63, 88, 101, 103 and 168 were obtained from the Department of Microbiology, University of Newcastle-upon-Tyne, U.K.; strains 297, 385, 417, 434, 436, 439, 461, 469, 478, 504 and 517 were obtained from the Department of Medicine, Charing Cross Hospital, London, U.K.; strains KS69, KS70, KS71, ER2, NK1 and T1 were obtained from the National Bacteriological Laboratory, Stockholm, Sweden; and strains KV, SC and WJ were isolated in our own laboratory. The bacteria were stored on slopes of Dorset egg medium and before use were subcultured on five consecutive days at 37 °C in static Oxoid nutrient broth no. 2 to obtain full expression of type 1 fimbriation. Silicized glassware was used for the subcultures to minimize attachment of the organisms to the walls of the containers.

Buffer systems. Phosphate-buffered saline (PBS) pH 6.8 was used in all adherence experiments; this contained (g l⁻¹): KH₂PO₄ 3.45, Na₂HPO₄ 4.45, NaCl 5.00 and KCl 0.20. When required, D-mannose was added at a concentration of 2.5% (w/v). PBS covering a range of pH values from 5.3 to 8.3 was used to determine the optimum pH for bacterial adherence. The buffer for the bioluminescence analysis was 25 mM-HEPES buffer pH 7.75 containing 2 mM-EDTA.

Extraction of bacterial ATP. ATP was extracted from suspensions of bacteria in PBS by vortex mixing 100 µl bacterial suspension with 100 µl Lumac NRB reagent (nucleotide releasing reagent for bacteria; Sterilin, U.K.) for 10 s followed by the immediate addition of 800 µl HEPES buffer (Fig. 1). A similar procedure was employed for the extraction of ATP from adherent bacteria in polystyrene tubes but using 40 µl of a mixture of equal volumes of NRB and PBS (NRB/PBS reagent) in order to maintain the same ratio of NRB:PBS that was present when extracting ATP from bacterial suspensions in PBS. These extracts were vortex mixed for 10 s and then treated with 160 µl HEPES buffer.

Bioluminescence ATP assay. Extracted ATP was measured using an LKB Luminometer 1250 after adding 100 µl LKB ATP-monitoring reagent (firefly luciferin/luciferase) to a 200 µl sample. Light emission was registered in mV on a digital display unit.

$$\text{Adherence ratio} = \frac{\text{Light emission (mV) from extract of adherent bacteria} \times 1000}{\text{Light emission (mV) from reference extract of PBS suspension} \times 15}$$

Fig. 1. Flow diagram of the bioluminescence/polystyrene tube adherence assay.

Polystyrene tubes. The tubes used for the adherence assay were those designed for use with the LKB Luminometer 1250 (available from Clinicon, U.K.), these being hydrophobic (non-wettable) polystyrene. A cross-hatching in the base of the tubes conveniently increased the internal surface area. All operations were performed in a single tube.

Adherence assay. A flow diagram of the bioluminescence/polystyrene tube adherence testing system is presented in Fig. 1. The fifth overnight subculture in static nutrient broth for each test organism was diluted 1/10 in PBS to yield a suspension containing about 1×10^8 c.f.u. ml⁻¹. Duplicate 300 µl samples from this suspension were placed into polystyrene tubes and incubated for 10 min in a waterbath at 37 °C. The bacterial suspensions were then aspirated using a water pump fitted with a trap containing disinfectant, and the tubes washed twice with 1 ml PBS delivered from a repeating dispenser. Adherence assays were set up and the polystyrene tubes subsequently washed at 1 min intervals so that up to 10 samples, each in duplicate, could be handled conveniently in any given experiment. Bacterial ATP was extracted and measured as described above.

Calculation of adherence ratio. The amount of ATP in a 200 µl sample of an extract of the original bacterial suspension in PBS served as the reference point for calculating the ratio of the number of bacteria adhering to the polystyrene tubes to the total number of organisms present in the PBS suspension. The number of adherent bacterial cells per thousand of the population in the original PBS suspension was calculated as follows:

$$\frac{\text{Mean mV reading of extract of bacteria adhering to tubes} \times 1000}{\text{mV Reading of extract of bacterial suspension in PBS} \times 15 \text{ (volume correction factor)}}$$

Effect of bacterial numbers on adhesion. Serial dilutions of broth cultures of four representative organisms (strains 23, 49, 56 and 297) were prepared in PBS containing 10% (v/v) nutrient broth and tested for adherence in the normal manner.

Effect of centrifugation and nutrient broth on adhesion. The third overnight subculture in static nutrient broth for each of six strains (23, 49, 56, 103, 168 and 297) was centrifuged for 15 min at 1400 g and the pellets were

resuspended in PBS and then diluted 1/10 in PBS containing 0%, 10% or 20% (v/v) nutrient broth. These dilutions were tested for adherence and the results compared with parallel cultures treated by the normal procedure.

Effect of bacterial growth phase on adhesion. Samples were taken at regular intervals from the fifth subculture in static nutrient broth for two representative strains (23 and 49). These samples were diluted 1/10 in PBS and tested for adhesion to polystyrene tubes by the normal procedure. The phase in the growth cycle was assessed by A_{560} .

Effect of inhibition of flagella synthesis on adhesion. Six adherent flagellate strains (12, 23, 168, 297, 385 and 504) were subcultured four times in static nutrient broth followed by a single subculture in broth containing 4 mM-EDTA to inhibit flagella synthesis (Weiser *et al.*, 1972). The ability of these organisms to adhere to polystyrene was then compared with that of parallel cultures grown in the absence of EDTA.

Effect of prolonging the assay incubation period on the adhesion of fimbriate, non-flagellate organisms. When broth cultures of bacteria which had been diluted 1/10 in PBS were incubated at 37 °C for more than 10 min an increase in extractable ATP was observed which was attributed to bacterial growth. This factor was allowed for whenever adhesion tests were performed using prolonged incubation periods by incubating samples of the bacterial suspensions in PBS under identical conditions to the test samples, and using the total extractable ATP level in these reference tubes as the denominator in the formula for calculating the adherence ratio. The adherence of four type 1 fimbriate but non-flagellate strains (434, 439, 517 and KS69) was measured using this procedure after 10, 60 and 150 min incubation, and two type 1 fimbriate and flagellate strains (297 and 504) were studied similarly after subculture in the presence and absence of 4 mM-EDTA.

Reference biological adherence methods. Human buccal cells and uroepithelial cells, obtained from healthy individuals, were used for comparison with the polystyrene tube method for adherence of the 34 test organisms. These cells were washed three times in PBS and then resuspended to a concentration of approximately 10^5 ml⁻¹. A mixture of 10^5 epithelial cells and 10^8 bacteria in 2 ml PBS was incubated with rotation for 1 h at 37 °C, and then vacuum filtered using a 5 µm Nuclepore polycarbonate membrane. The filter pad was washed and pressed on to a glass microscope slide, and the resulting imprint was stained and examined microscopically. The mean number of bacteria adhering per cell was determined from the counts for 30 epithelial cells, and adherence to the uromucoid, which was always present in preparations of uroepithelial cells, was noted simultaneously. Adherence to mucoid could not be quantified, but since organisms which adhered to mucoid did so in very large numbers, a score of 1 was given to those strains which showed such definite attachment, and a score of 0 was given to those which did not, in order to allow statistical analysis of the observations. Full details of these reference methods have been published in a separate communication (Chick *et al.*, 1981).

Comparison of polystyrene tube method with reference biological methods. The 34 test strains of *E. coli* were examined after five consecutive daily subcultures in nutrient broth for adherence of each to polystyrene, buccal cells, uroepithelial cells and uromucoid, and also for agglutination of guinea-pig and human erythrocytes. All reactions were performed both in the presence and absence of 2.5% D-mannose. Samples were also taken simultaneously for morphological inspection by electron microscopy.

Electron microscopy. Pellets of each organism were resuspended in a stain of 2% (w/v) phosphotungstic acid containing 0.2% (w/v) BSA and adjusted to pH 7.0 with NaOH. The bacteria were then taken on to carbon-coated collodion films on copper grids and examined using a Philips 300 electron microscope. All cultures contained a mixture of fimbriate and non-fimbriate cells and the percentage of cells with fimbriae was noted in each case. Most cultures also contained bacterial cells which possessed flagella, and the percentage of cells with flagella was recorded similarly for each organism. All these observations were made independently by the microscopist.

Haemagglutination reactions. The ability of the organisms to agglutinate guinea-pig erythrocytes (inhibited by D-mannose), or human erythrocytes in the presence of D-mannose, was used to differentiate between possession of MS type 1 fimbriae and MR fimbriae, respectively, by strains for which fimbriae could be detected by electron microscopy. Erythrocytes from citrated whole blood were in each case washed twice with PBS pH 6.8 and resuspended in this buffer to a concentration of 3% (v/v). One drop was then mixed with one drop of a suspension of 10^9 bacteria ml⁻¹ in PBS alone or PBS containing 2.5% D-mannose on a glass slide with gentle rocking at ambient temperature (about 20 °C). Agglutination was observed visually and scored as described in Table 2.

RESULTS

Precision, recovery and sensitivity data. The intra-assay precision for the polystyrene tube adhesion testing method expressed in terms of coefficient of variation for the analysis of 12 replicate samples was $\pm 8.5\%$, while the recovery of 10 pmol of exogenous ATP per tube was $104 \pm 3\%$ ($n = 12$). The mean coefficient of variation for the analysis of the adhesion of 21 strains on each of three consecutive days when full expression of type 1 fimbriation appeared to have been achieved (the third, fourth and fifth subcultures in static nutrient broth) was $\pm 36.3\%$. The

Fig. 2. Effect of pH on the adhesion of six strains of *E. coli* to polystyrene. The strains used were: 297 (●), 23 (○), 103 (■), 49 (□), 56 (▲) and 44 (△).

Fig. 3. Effect of incubation time and temperature on the adherence of one representative *E. coli* strain (436). The assay tubes were incubated at 4 °C (▲), 20 °C (●), 37 °C (■) and 56 °C (◆).

limit of detection for the bioluminescence assay was 0.02 pmol ATP in a 200 µl sample, this being equivalent to about 5×10^5 *E. coli* ml⁻¹ PBS suspension or about 1×10^4 adherent bacteria per tube.

Effect of pH and phosphate on adhesion. The optimum pH for the adherence of strongly adherent strains of *E. coli* was 6.8, while weakly adherent strains were unaffected by variations in pH (Fig. 2). Phosphate *per se* had no influence on the adhesion process.

Effect of temperature on adhesion. Adherence to polystyrene increased slowly but steadily on prolonged incubation at 4 °C, and the rate of attachment increased with temperature. However, adherence was completely inhibited after a few minutes incubation at 56 °C, and an incubation temperature of 37 °C appeared to be optimal. Data for an organism possessing both type 1 fimbriae and flagella (strain 436) are illustrated in Fig. 3.

Effect of bacterial numbers on adhesion. For any investigated strain, almost identical adherence ratios were obtained in adherence assays using PBS suspensions containing from 6×10^6 to 5×10^8 c.f.u. ml⁻¹. Data for the four strains tested were applied to a modification of the Langmuir adsorption isotherm (Fletcher, 1977) but were found not to fit the isotherm equation.

Effect of centrifugation and nutrient broth on adhesion. Centrifugation of cultures produced a detrimental effect on bacterial adhesion, reducing the adhesive capacity of the six strains tested by 40–70% (Table 1). This was the case irrespective of whether the centrifuged pellets were

Table 1. *Adherence ratios for six E. coli strains before and after centrifugation*

Treatment of bacterial cells	Adherence ratios of strains of <i>E. coli</i>						Difference from control*
	Strain 23	Strain 49	Strain 56	Strain 103	Strain 168	Strain 297	
Uncentrifuged control; diluted in PBS	38	45	29	9	17	37	—
Centrifuged and resuspended in broth; diluted in PBS	25	14	5	7	9	26	$P < 0.02$
Centrifuged and resuspended in PBS; diluted in PBS	7	17	12	3	7	12	$P < 0.01$
Centrifuged and resuspended in PBS; diluted in 10% broth	21	12	11	5	6	26	$P < 0.02$
Centrifuged and resuspended in PBS; diluted in 20% broth	22	9	6	5	7	25	$P < 0.02$

* Adherence ratios for each centrifuged treatment group were compared with those of the uncentrifuged control group using Student's paired *t*-test. This experiment was performed with the third overnight subculture of each strain in static nutrient broth in which expression of type 1 fimbriae was sub-maximal for strains 103 and 297.

resuspended in nutrient broth or PBS, the former producing no significant influence on adhesion even when present in the final incubation suspension at a concentration of 20%.

Effect of bacterial growth phase on adhesion. The ability of strains possessing both type 1 fimbriae and flagella to adhere to polystyrene increased during exponential growth to reach a peak towards the end of the exponential phase, and declined markedly in the stationary phase. However, organisms lacking either type 1 fimbriae or flagella or both were weakly adherent irrespective of the phase of growth.

Adherence ratios and effect of D-mannose on adhesion. Adherence data and other relevant observations are presented in Table 2. A variation of more than 200-fold was observed in the adherence ratios of the 34 test strains when assayed for adherence to polystyrene, even in the stationary phase of growth, and two groups could be identified. Sixteen strains with an adherence ratio < 10 were classified as being weakly adherent, while the remaining 18 strains which showed an adherence ratio ≥ 10 were designated strongly adherent, this adherence being strongly inhibited by 2.5% D-mannose. The mannose sensitivities for the weakly adherent group could not be determined accurately due to the low level of adherence, but about half of these strains appeared to be adherent in a mannose-sensitive manner and half in a mannose-resistant manner.

Role of fimbriae and flagella in adhesion. Bacterial adhesion to polystyrene showed no association with the presence of MR fimbriae as assessed by the agglutination of human erythrocytes in the presence of mannose (Table 3). However, all 18 strains which were strongly adherent produced mannose-sensitive haemagglutination of guinea-pig erythrocytes, and since electron microscopy showed all these strains to be fimbriate they were judged to possess MS fimbriae. Electron microscopy also revealed that a major proportion of individual cells ($\geq 40\%$) for all but one of the 18 organisms possessed flagella as well as fimbriae. Conversely eight other MS fimbriate strains which showed only weak adhesion to polystyrene were sparsely flagellate or lacked these organelles entirely (Table 3).

Effect of inhibition of flagella synthesis on adhesion. Electron microscopy confirmed that EDTA-treated cultures were non-flagellate. The absence of flagella was associated with virtual abolition of the ability of the organisms to adhere to polystyrene (Fig. 4). Fimbrial synthesis was unaffected by growth in EDTA broth since all strains still produced strong agglutination of guinea-pig erythrocytes, while EDTA *per se* had no inhibitory effect in the adhesion assay.

The adherence of non-flagellate strains increased on prolonged incubation but still remained at a low level in comparison to values recorded for flagellate strains (Table 4).

Comparison of the polystyrene tube method with reference biological methods. All but one of the 34 strains of *E. coli* showed a significant level of adherence to buccal epithelial cells; data for adherence to uroepithelial cells have been published elsewhere (Chick *et al.*, 1981). There was no association between adherence to either buccal or uroepithelial cells and possession of flagella ($P > 0.1$, Mann-Whitney *U* test). Furthermore, there was no correlation between attachment

Table 2. *Haemagglutination reactions, electron microscopy observations and adherence data for 34 strains of E. coli*

Strain no.	Haemagglutination reactions*			Adherence to polystyrene		Adherence to uromucoid without D-mannose‡
	Guinea-pig erythrocytes without D-mannose	Human erythrocytes with D-mannose	Flagella present (EM observation)†	Ratio without D-mannose	Ratio with D-mannose	
7	—	++	—	1	1	0
10	—	—	—	5	2	0
12	+++	—	+	41	2	1
20	—	—	—	3	4	0
23	+++	—	+	83	9	1
44	+++	—	—	5	1	1
49	+++	+++	+	29	3	1
56	+++	+++	+	21	2	0
57	—	+++	—	2	2	0
63	+++	+++	+	137	9	1
88	—	+++	—	3	3	0
101	—	+	+	4	3	0
103	+++	++	+	38	4	1
168	+++	—	+	24	4	1
297	+++	—	+	176	5	1
385	+++	+	+	11	3	1
417	+++	+	—	19	1	1
434	+++	+	—	2	1	0
436	+++	+++	+	19	1	1
439	+++	—	—	1	1	1
461	+++	—	+	17	3	1
469	+++	+	+	50	3	1
478	+++	+	—	3	1	1
504	+++	—	+	65	4	1
517	+++	+	—	5	2	1
KS69	+++	++	—	3	1	1
KS70	+++	+	+	41	5	1
KS71	+++	+++	+	12	2	1
ER2	+++	+	—	2	1	1
NK1	+++	+++	—	2	0	1
T1	—	+	—	5	4	0
KV	+++	—	+	13	4	1
SC	—	+++	—	2	3	0
WJ	+++	++	+	25	2	1

* Symbols: + + +, rapid, strong reaction; + +, moderate reaction; +, slow, weak reaction; and —, no observed haemagglutination.

† All strains possessed fimbriae except numbers 7 and 20. Strains were scored + for flagella if $\geq 40\%$ of individual cells were flagellate, and — if $< 40\%$ of individual cells were flagellate.

‡ A score of 1 was given to strains which showed definite attachment to uromucoid and a score of 0 to strains which did not.

Table 3. *Analysis of the adherence of 34 strains of E. coli to polystyrene tubes*

Sub-group	No. of strains	No. showing strong adherence (ratio ≥ 10)	Median adherence ratio	Statistical analysis on individual values*
MR fimbriae present	23	11	5	$P > 0.1$
MR fimbriae absent	11	7	17	
MS fimbriae present	26	18	19	$P < 0.01$
MS fimbriae absent	8	0	3	
Flagellate cells abundant ($\geq 40\%$)	18	17	27	$P < 0.001$
Flagellate cells sparse or absent ($< 40\%$)	16	1	3	
MS fimbriae present and flagella abundant	17	17	29	$P < 0.001$
MS fimbriae absent and flagella sparse or absent	17	1	3	

* Adherence data were analysed by the Mann-Whitney *U* test.

Fig. 4. Abolition of the adhesion of six strains of *E. coli* following subculture in nutrient broth containing 4 mM-EDTA.

Table 4. *Adherence ratios following prolonged incubation of non-flagellate strains of E. coli in polystyrene tubes*

Values in parentheses indicate adherence ratios recorded after growth in nutrient broth containing 4 mM-EDTA to inhibit flagella synthesis.

Strain type	Strain no.	Incubation time (min)		
		10	60	150
Non-flagellate strains	KS69	0	3	—
	434	0	3	—
	439	1	4	8
	517	5	9	7
Flagellate controls	297	142 (2)	266 (7)	293 (18)
	504	58 (2)	63 (9)	73 (25)

of the strains to polystyrene and adhesion to either buccal cells or uroepithelial cells, this being the case irrespective of whether the whole series of organisms was considered or only the 18 strains which showed strong adherence to polystyrene ($P > 0.1$, Mann-Whitney *U* test).

The individual adherence ratios obtained in the polystyrene tube assay for strains which adhered to uromucoid were compared with the ratios for strains which did not, using the Mann-Whitney *U* test. The median values were 19.1 and 2.6 for the mucoid-adherent and non-adherent groups respectively, and the analysis indicated a significant association between adherence to polystyrene and adherence to mucoid ($P < 0.002$).

A correlation was found between bacterial adherence to uromucoid and possession of flagella ($P < 0.02$, Mann-Whitney *U* test), but this appeared to be an epiphenomenon since there was also a significant association between possession of flagella and possession of MS fimbriae ($P < 0.05$, χ^2 test) which indicated that expression of these surface characteristics was coincidental.

As reported previously (Chick *et al.*, 1981) adherence to uromucoid was significantly associated with MS fimbriation, 24 of 26 MS fimbriate strains adhering to mucoid whereas all strains without MS fimbriae were non-adherent ($P < 0.001$, Mann-Whitney *U* test). A total of 17 of the 24 strains which adhered to mucoid also adhered strongly to polystyrene. All but one of these 17 possessed flagella as well as MS fimbriae, while the remaining seven strains which adhered to mucoid but which were only weakly adherent to polystyrene lacked flagella. One anomalous strain with both MS fimbriae and flagella adhered to polystyrene but not to uromucoid.

DISCUSSION

The bioluminescence assay presented above provides a rapid and sensitive method for quantifying bacterial adherence to solid surfaces. A very wide variation was observed in the ability of different strains of *E. coli* to adhere to polystyrene, and a number of important factors have been identified which affect the adhesive characteristics of bacteria in this test system. Although the observed inter-assay variation was high ($\pm 36.3\%$) this did not affect the classification of any of the strains as strongly or weakly adherent. It is considered that the day to day variation in adherence ratio may be attributed largely to variability in the expression of surface bacterial characteristics in different broth cultures. Maximum expression of type 1 fimbriae appeared to be achieved after three to five subcultures in static nutrient broth, but electron microscopy revealed that the percentage of fimbriate cells for each strain could vary from day to day even after such repeated subculture. The high inter-assay variation does not indicate poor reproducibility of the adherence assay *per se*, since the intra-assay variation was relatively low ($\pm 8.5\%$).

The rate of attachment of bacteria to the side-walls and base of a polystyrene tube might be expected to be governed largely by Brownian movement and sedimentation resulting in chance interaction of the cells with the polystyrene surface, while attachment *per se* might be expected to be mediated largely by non-specific processes of adsorption. However, data for the adherence of four strains which possessed both type 1 (MS) fimbriae and flagella tested over a wide range of dilutions failed to fit a modification of the Langmuir adsorption isotherm. This suggested that biological phenomena are important in determining the rate of adhesion instead of, or in addition to, physico-chemical factors.

One factor which is well documented as playing a major role in the adherence of Gram-negative bacteria to solid surfaces is the possession of fimbriae. No association was found between adherence to polystyrene and possession of MR fimbriae, but there was a very significant correlation between adherence data and possession of type 1 (MS) fimbriae. Type 1 fimbriate *E. coli* attached strongly to polystyrene tubes in a mannose-sensitive manner, whereas all strains devoid of type 1 fimbriae were weakly adherent.

It was apparent that other factors must be involved with adhesion in addition to type 1 fimbriae since eight strains which possessed these organelles showed only a low level of adherence. The explanation for this finding came from the observation that cultures of all organisms showing strong adherence to polystyrene contained a high proportion of cells which possessed flagella as well as type 1 fimbriae. With only one exception both types of organelle were found to be required for a high level of attachment. The essential involvement of flagella in the adhesion process was confirmed by the fact that adherent strains could be rendered non-adherent simply by inhibiting flagella synthesis by growth in EDTA broth. Damage to or loss of flagella is the most likely explanation for the marked detrimental effect which centrifugation of broth cultures had on adhesion, free flagella being a common finding during electron microscopic examination of centrifuged bacterial cultures. Furthermore, the effects of variation in growth phase on adhesion may also be largely attributable to changes in the motility of cells during growth in batch culture since motility is likely to decline with the onset of stationary phase (Fletcher, 1977, 1979).

Motility may be involved with adhesion in three possible ways. Firstly, it may simply increase the chances of a bacterial cell coming into contact with the attachment surface; secondly, motility may enable bacteria to overcome the electrostatic potential barrier which exists between surfaces of like charge; thirdly, flagella *per se* may be directly involved in mediating attachment. Guentzel & Berry (1975) considered that the most likely explanation for the association they observed between virulence of *Vibrio cholerae* and motility was the increased chance of association between bacterial cells and the intestinal mucosa. Conversely, Fletcher (1979) speculated that adherence of a motile *Pseudomonas* sp. to polystyrene might be facilitated by an ability to overcome electrostatic forces of repulsion, while there is some evidence for a direct holdfast role for flagella in the attachment of Gram-negative bacilli (Meadows, 1971) and vibrios (de Boer *et al.*, 1975) to solid surfaces. In the present study a small increase in adherence was observed with non-flagellate or de-flagellated strains of *E. coli* on prolonged incubation in

polystyrene tubes, but the level of adhesion remained low in comparison with that of the flagellate strains. This would suggest that flagella play a more active role in the attachment process than simply increasing the statistical chance of cell-surface contact.

Flagella and fimbriae are both proteinaceous structures and the dramatic inhibition of adhesion produced at 56 °C is almost certainly due to protein denaturation in either or both of these types of organelle. Active motility is an energy-requiring process, hence 37 °C was found to be the optimum temperature for adherence. However, the fact that a slow but progressive increase in adherence was observed at 4 °C suggests that the forces of attraction which mediate attachment once cell-surface contact has been established are not dependent on expenditure of energy. It has been suggested that hydrophobic interactions associated with masking of cell surface potential by fimbriae might influence the adherence characteristics of enteropathogenic strains of *E. coli* (Smyth *et al.*, 1978). Hydrophobic interactions are likely to be involved with bacterial adhesion to polystyrene, but it would appear that intimate cell-surface contact must first be promoted through the action of flagella.

No correlation was found between adhesion of *E. coli* to polystyrene and adhesion to uroepithelial or buccal epithelial cells. This was not a surprise finding, however, since attachment to polystyrene was observed to be mediated by type 1 (MS) fimbriae whereas adherence to human uroepithelial cells is mediated by MR fimbriae (Ørskov *et al.*, 1980a; Chick *et al.*, 1981). Buccal cells appear to provide a relatively non-specific attachment surface compared with uroepithelial cells or polystyrene, only one of the 34 test strains being non-adherent in this system. One obvious difference in the attachment of bacteria to the biological cell surfaces compared with attachment to polystyrene is that adherence is maximal with stationary phase cultures using epithelial cells, whereas maximum adherence to polystyrene is achieved with bacteria in the exponential growth phase. The most likely explanation for this difference lies with the involvement of bacterial motility in adhesion, flagella being an essential factor when polystyrene is the attachment surface but of no importance with the three biological assays. However, it is of interest to note in this context that motility has been reported recently to promote the attachment of *Salmonella typhimurium* to HeLa cells (Jones *et al.*, 1981).

Bacterial adhesion to both polystyrene and uromucoid was mediated by type 1 (MS) fimbriae, and a significant association was observed between adherence of our *E. coli* strains to these two surfaces ($P < 0.002$). However, this correlation masked one important difference in that whereas 24 of the 26 strains with type 1 fimbriae adhered to uromucoid, only those strains possessing both type 1 fimbriae and flagella (with one exception) adhered to polystyrene. This distinction was still apparent even when the incubation period for the polystyrene tube assay was extended to 1 h or more. Hence the polystyrene tube method may give an indication of the susceptibility of motile type 1 fimbriate bacteria to be trapped by uromucoid, but it would appear to have no application to non-motile organisms. However, it could be of interest to determine whether or not there is any correlation between the adherence of pathogenic bacteria to polystyrene and their susceptibility to phagocytosis, since type 1 fimbriae are capable of mediating attachment of non-opsonized bacteria to the surface membranes of phagocytic cells (Silverblatt *et al.*, 1979; Mangan & Snyder, 1979; Bar-Shavit *et al.*, 1980), while a recent report has demonstrated bacterial motility to be an important factor in the phagocytosis of a variety of Gram-negative bacilli by murine macrophages (Tomita *et al.*, 1981). Furthermore, it is considered that the bioluminescence detection system described in this communication could be applied to the study of mechanisms of bacterial adhesion using certain biological as well as non-biological attachment surfaces, or with polystyrene coated with appropriate 'receptor' substances.

We are grateful to Professor M. Sussman, Department of Microbiology, University of Newcastle-upon-Tyne, Dr A. P. Roberts, Department of Medicine, Charing Cross Hospital, London and Dr G. Källénus, National Bacteriological Laboratory, Stockholm, Sweden for kindly supplying test organisms used in this study.

REFERENCES

- ARONSON, M., MEDALIA, O., SCHORI, L., MIRELMAN, D., SHARON, N. & OFEK, I. (1979). Prevention of colonization of the urinary tract of mice with *Escherichia coli* by blocking of bacterial adherence with methyl α -D-mannopyranoside. *Journal of Infectious Diseases* **139**, 329–332.
- BAR-SHAVIT, Z., GOLDMAN, R., OFEK, I., SHARON, N. & MIRELMAN, D. (1980). Mannose-binding activity of *Escherichia coli*: a determinant of attachment and ingestion of the bacteria by macrophages. *Infection and Immunity* **29**, 417–424.
- BRINTON, C. C., JR (1965). The structure, function, synthesis and genetic control of bacterial pili and a molecular model for DNA and RNA transport in Gram-negative bacteria. *Transactions of the New York Academy of Sciences* **27**, 1003–1054.
- CHICK, S. & ASSCHER, A. W. (1980). Studies on the significance of bacterial adherence in experimental urinary tract infection. In *Pyelonephritis, vol. IV, Urinary Tract Infections*, pp. 67–71. Edited by H. Losse, A. W. Asscher & A. E. Lison. Stuttgart & New York: Georg Thieme Verlag.
- CHICK, S., HARBER, M. J., MACKENZIE, R. & ASSCHER, A. W. (1981). Modified method for studying bacterial adhesion to isolated uroepithelial cells and uromucoid. *Infection and Immunity* **34**, 256–261.
- DE BOER, W. E., GOLTEN, C. & SCHEFFERS, W. A. (1975). Effects of some physical factors on flagellation and swarming of *Vibrio alginolyticus*. *Netherlands Journal of Sea Research* **9**, 197–213.
- DISTLER, W., KRÖNCKE, A. & MAURER, G. (1980). Adenosine triphosphate content of human dental plaque as a measure of viable cell mass. *Caries Research* **14**, 265–268.
- FADER, R. C., AVOTS-AVOTINS, A. E. & DAVIS, C. P. (1979). Evidence for pili-mediated adherence of *Klebsiella pneumoniae* to rat bladder epithelial cells in vitro. *Infection and Immunity* **25**, 729–737.
- FLETCHER, M. (1976). The effects of proteins on bacterial attachment to polystyrene. *Journal of General Microbiology* **94**, 400–404.
- FLETCHER, M. (1977). The effects of culture concentration and age, time, and temperature on bacterial attachment to polystyrene. *Canadian Journal of Microbiology* **23**, 1–6.
- FLETCHER, M. (1979). The attachment of bacteria to surfaces in aquatic environments. In *Adhesion of Micro-organisms to Surfaces*, pp. 87–108. Edited by D. C. Ellwood, J. Melling & P. Rutter. London, New York & San Francisco: Academic Press.
- GUENTZEL, M. N. & BERRY, L. J. (1975). Motility as a virulence factor for *Vibrio cholerae*. *Infection and Immunity* **11**, 890–897.
- HARBER, M. J. (1982). Applications of luminescence in medical microbiology and haematology. In *Clinical and Biochemical Luminescence*, pp. 189–218. Edited by L. J. Kricka & T. J. N. Carter. New York: Marcel Dekker.
- HARBER, M. J., CHICK, S., MACKENZIE, R. & ASSCHER, A. W. (1982a). Lack of adherence to epithelial cells by freshly isolated urinary pathogens. *Lancet* **i**, 586–588.
- HARBER, M. J., MACKENZIE, R. K. & ASSCHER, A. W. (1982b). Bacterial adherence and the urinary tract. *Lancet* **i**, 1352–1353.
- JONES, G. W., RICHARDSON, L. A. & UHLMAN, D. (1981). The invasion of HeLa cells by *Salmonella typhimurium*: reversible and irreversible bacterial attachment and the role of bacterial motility. *Journal of General Microbiology* **127**, 351–360.
- KÄLLENUS, G., MÖLLBY, R., SVENSON, S. B., WINBERG, J. & HULTBERG, H. (1980). Identification of a carbohydrate receptor recognized by uropathogenic *Escherichia coli*. *Infection* **8** (supplement 3), S288–S293.
- KÄLLENUS, G., MÖLLBY, R., SVENSON, S. B., HELIN, I., HULTBERG, H., CEDERGREN, B. & WINBERG, J. (1981). Occurrence of P-fimbriated *Escherichia coli* in urinary tract infections. *Lancet* **ii**, 1369–1372.
- LEFFLER, H. & SVANBORG EDÉN, C. (1980). Chemical identification of a glycosphingolipid receptor for *Escherichia coli* attaching to human urinary tract epithelial cells and agglutinating human erythrocytes. *FEMS Microbiology Letters* **8**, 127–134.
- MANGAN, D. F. & SNYDER, I. S. (1979). Mannose-sensitive interaction of *Escherichia coli* with human peripheral leukocytes in vitro. *Infection and Immunity* **26**, 520–527.
- MEADOWS, P. S. (1971). The attachment of bacteria to solid surfaces. *Archiv für Mikrobiologie* **75**, 374–381.
- ØRSKOV, I., ØRSKOV, F. & BIRCH-ANDERSON, A. (1980a). Comparison of *Escherichia coli* fimbrial antigen F₇ with type 1 fimbriae. *Infection and Immunity* **27**, 657–666.
- ØRSKOV, I., FERENCZ, A. & ØRSKOV, F. (1980b). Tamm-Horsfall protein or uromucoid is the normal urinary slime that traps type 1 fimbriated *Escherichia coli*. *Lancet* **i**, 887.
- ROBRISH, S. A., KEMP, C. W. & BOWEN, W. H. (1978). Use of extractable adenosine triphosphate to estimate the viable cell mass in dental plaque samples obtained from monkeys. *Applied and Environmental Microbiology* **35**, 743–749.
- RUTTER, P. R. & ABBOTT, A. (1978). A study of the interaction between oral streptococci and hard surfaces. *Journal of General Microbiology* **105**, 219–226.
- SILVERBLATT, F. J., DREYER, J. S. & SCHAUER, S. (1979). Effect of pili on susceptibility of *Escherichia coli* to phagocytosis. *Infection and Immunity* **24**, 218–223.
- SMYTH, C. J., JONSSON, P., OLSSON, E., SÖDERLIND, O., ROSENGREN, J., HJERTÉN, S. & WADSTRÖM, T. (1978). Differences in hydrophobic surface characteristics of porcine enteropathogenic *Escherichia coli* with or without K88 antigen as revealed by hydrophobic interaction chromatography. *Infection and Immunity* **22**, 462–472.
- TOMITA, T., BLUMENSTOCK, E. & KANEGASAKI, S. (1981). Phagocytic and chemiluminescent responses of mouse peritoneal macrophages to living and killed *Salmonella typhimurium* and other bacteria. *Infection and Immunity* **32**, 1242–1248.
- VÄISÄNEN, V., ELO, J., TALLGREN, L. G., SITONEN, A., MÄKELÄ, P. H., SVANBORG EDÉN, C., KÄLLENUS, G., SVENSON, S. B., HULTBERG, H. & KORHONEN, T. (1981). Mannose-resistant haemagglutination and P antigen recognition are characteristic of *Escherichia coli* causing primary pyelonephritis. *Lancet* **ii**, 1366–1369.

- WEISER, R., MOFFAT, D. B., WIMPENNY, J. C., ASSCHER, A. W. & SUSSMAN, M. (1972). Ethylenediamine tetra-acetic acid (EDTA) induced morphological changes in *Proteus mirabilis*. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene (Abteilung I, Originale A: Medizinische Mikrobiologie und Parasitologie)* **222**, 52-56.