THE FIMBRIAL ANTIGENS OF *SHIGELLA FLEXNERI*

BY R. R. GILLIES AND J. P. DUGUID

Bacteriology Department, University of Edinburgh

(With Plates 2–4)

INTRODUCTION

Bacterial fimbriae are filamentous appendages which are smaller and more numerous than flagella, and unrelated to motility. They are demonstrable only by electron-microscopy and were first clearly described by Houwink & van Iterson (1950), who suggested that they may act as organs of attachment. Evidence of their adhesive properties was afforded by the observation of Duguid, Smith, Dempster & Edmunds (1955) that fimbriate strains of *Bacterium coli* adhered to and agglutinated red blood corpuscles, while most non-fimbriate strains did not. A similar correlation between fimbriation and haemagglutinating activity was later found in *B. cloacae* (Constable, 1956) and *Shigella flexneri* (Duguid & Gillies, 1956, 1957).

Duguid *et al.* (1955) observed that haemagglutination by fimbriate *coli* strains was inhibited on mixing with antisera prepared by injection of living bacilli, though not with antisera prepared against bacilli heated at 100° C. for 2½ hr.; this suggested that fimbriae bear heat-labile antigens. Duguid & Gillies (1956) confirmed the antigenicity of the fimbriae of *Sh. flexneri*, showing that the fimbrial antibodies both agglutinated the bacilli and inhibited their haemagglutinating activity. *Sh. flexneri* readily undergoes a reversible mutation between a fimbriate, haemagglutinating phase (Pl. 2, fig. 1) and a non-fimbriate, non-haemagglutinating phase. Virtually pure cultures of the non-fimbriate phase are obtained by serial aerobic cultivation on agar plates and predominantly fimbriate cultures by serial aerobic cultivation in tubes of broth. These circumstances make possible the preparation of a 'pure fimbrial antiserum'. A crude fimbrial antiserum is first prepared by immunising a rabbit with a live, fimbriate-phase culture and this is absorbed with a non-fimbriate phase culture of the same strain so as to remove all antibodies for the somatic antigens. The agglutinating and haemagglutination-inhibiting activities remaining in the absorbed serum are ascribed with certainty to fimbrial antibodies. The present paper describes an investigation of the nature of the fimbrial antigens of *Sh. flexneri* and of their relationships with those of *Bact. coli* and other Gram-negative bacilli.

MATERIALS AND METHODS

Strains. We examined the *Sh. flexneri* strains obtained from the National Collection of Type Cultures (NCTC) and others recently isolated from dysentery cases, described by Duguid & Gillies (1957). The antisera mainly used were prepared against five strains, respectively, of O-serotype 1*a* (NCTC 8192), Type 2*b*

(NCTC 8518), Types 3 and 4*a* (strains recently isolated in Edinburgh) and Type 5 (NCTC 8524). The *Bact. coli* strains examined were those described by Duguid *et al.* (1955) and some additional haemolytic strains with similar biochemical reactions.

Culture methods. Fimbriate-phase cultures were obtained by making several (e.g. 3–6) serial aerobic cultivations at 37° C. for 48 hr. in cotton-stoppered tubes of nutrient broth, or, when large amounts of bacilli were required, in shallow layers of broth in large flat bottles (see Duguid & Gillies, 1957). Non-fimbriate-phase cultures were obtained by making several serial aerobic cultivations at 37° C. for 24–48 hr. on thin, well-dried nutrient agar plates. Before use, the culture was tested for haemagglutinating activity to confirm that it was fully fimbriate or purely non-fimbriate.

Haemagglutination tests. Red cells were separated by centrifugation from citrated guinea-pig blood, washed twice in 0·85 % NaCl solution and made up to a 3 % suspension in such saline. The tests were made in depressions on a white porcelain tile. A drop of red-cells was mixed with a drop of broth culture, centrifuged broth-culture deposit or surface growth from agar, and the tile rocked to and fro at room temperature for at least 5 min. The haemagglutination reaction was designated + + + + when coarse clumping commenced within a few seconds, + + + when moderate clumping commenced within ½ min., + + when fine clumping appeared only after 1 min., and + when very fine granules appeared after 3–5 min. (Pl. 2, fig. 2). Full fimbriation was indicated when a drop of uniformly mixed, uncentrifuged broth culture gave a + + or + + + reaction. Purely non-fimbriate cultures on agar gave no reaction, even when a large loopful of the solid growth material was mixed with the red cells.

Haemagglutination-inhibition tests. A drop of undiluted antiserum was mixed with the drop of red cells on the tile. A drop of bacillary suspension was then added and the resulting haemagglutination reaction compared with that in a parallel control test in which saline was substituted for the antiserum. The bacillary suspension was prepared in such strength as to give a + + + reaction in the control test.

Antisera were produced in adult rabbits by injection of living *Sh. flexneri* suspended in 0·85 % NaCl solution to a density of about $1{\cdot}5 \times 10^9$ bacilli per ml. Separate sera were prepared against fully fimbriate broth cultures and purely non-fimbriate agar cultures. In each case six injections were given at 3-day intervals, each injection equalling 0·25 ml., except the last (0·5 ml.); the first two injections were given subcutaneously and the remainder intravenously. Bleedings were made from the marginal ear vein 11 days after the last injection; after separating the serum, sodium azide was added to 0·08 %. Sera of especially high titre (e.g. 122,880 as in Table 5) were obtained after two further intravenous injections of 1 ml.

Agglutination tests were carried out in round-bottom tubes ($3 \times \frac{1}{2}$ in.) by a doubling-dilution method (Mackie & McCartney, 1956) and were incubated in a 37° C. water bath for 4 hr. After reading, the tests were stored at 4° C. for 12–18 hr. and read again. Titres throughout are stated as reciprocals of the highest dilution of the serum giving visible naked-eye agglutination.

Agglutinin absorption. This was done either with packed, washed, living bacilli or bacilli disrupted by heating the washed suspension for 1 hr. at 100° C. The final absorbing agent in each case was the deposit at the bottom of the centrifuge tube from which all excess saline had been removed. The serum to be absorbed was added to the bacterial mass which was then emulsified in the serum by means of a sterile glass rod. The mixture was held at 37° C. for 1 hr. after which it was centrifugalized until the supernatant serum was quite clear. By this method any significant dilution of the serum was avoided.

Electron microscopical observations of shadow cast films of bacilli were made as described by Duguid & Gillies (1957).

RESULTS

Properties of crude fimbrial antiserum

Blood serum samples were taken from rabbits before immunization; in tube-agglutination tests these showed the presence of natural antibodies for fimbriate *Sh. flexneri* at low titres only (< 30, 30 or 60). The animals were then immunized by injection of living fimbriate *flexneri* cultures. The resulting 'crude fimbrial antisera' showed high agglutinin titres (1,920 to 122,880) for living fimbriate bacilli, equally for those of homologous and heterologous O-serotypes. The fimbrial agglutination reaction closely resembled flagellar agglutination; it was rapid and gave a bulky, loosely floccular deposit which readily dispersed into fine granules on shaking. The clumping became visible in 15–30 min. at 37° C. and was maximal at 2 hr.; only rarely was any further increase of titre noted after overnight storage at 4° C. When the crude fimbrial antisera were tested against bacilli from non-fimbriate-phase cultures, they gave the slow, compact small-flake clumping that is typical of somatic agglutination involving the type-specific O-antigen. Their O-agglutinin titres for the non-fimbriate bacilli were much lower than their fimbrial agglutinin titres, being from 240 to 1920 for strains of the homologous O-serotype and from < 30 to 480 for strains of heterologous O-serotypes. The presence of O-antibodies in the fimbrial antisera indicates that the fimbriate bacilli used for immunization were not deficient in O-antigen.

Influence of fimbriation on O-agglutinability

Other rabbits were immunized with living non-fimbriate-phase cultures of the same strains as used to produce the fimbrial antisera. The resulting 'crude non-fimbrial antisera' were tested against living bacilli from non-fimbriate-phase cultures; they gave compact somatic agglutination at high titres (1920 to 15,360) with strains of the homologous O-serotype, and at lower titres (120 to 7680) with strains of heterologous O-serotypes. When tested against fimbriate bacilli, the non-fimbrial antisera gave a modified somatic agglutination reaction with formation of very fine granules adhering to the walls of the tube. The agglutinin titres shown by non-fimbrial antisera for living fimbriate bacilli of the homologous O-serotype were generally much lower (e.g. 16–64-fold lower) than those shown for living non-fimbriate bacilli of the same strain. In some cases, identical titres were obtained

for the fimbriate and the non-fimbriate bacilli, but agglutination of the former was weak and incomplete in the higher serum dilutions.

These findings suggest that fimbriae confer relative O-inagglutinability by partially covering or masking the O-antigens, and so act in the same way as the K-antigens in *Bacterium coli* (Kauffmann, 1947); complete O-inagglutinability, such as is found in a 'fully-Vi' culture of *Salm. typhi*, was not encountered. The fimbriate bacilli could be rendered fully O-agglutinable through removal of the fimbriae by boiling at 100° C. for 1 hr. (see below). For example, an antiserum prepared against non-fimbriate bacilli of a Type 5 strain gave O-agglutinin titres of 7680 and 3840, respectively, with the living and boiled non-fimbriate bacilli, a titre of only 240 with living fimbriate bacilli of the same strain, but one of 3840 with these fimbriate bacilli after boiling and washing by centrifugation.

Properties of pure fimbrial antiserum

The mutability of fimbriation in *Sh. flexneri* was utilized to prepare 'pure fimbrial antiserum', by absorption of a crude antiserum, obtained from a rabbit immunized with a fimbriate-phase broth culture, with a non-fimbriate-phase agar culture of the same strain. To obtain 20 ml. of absorbed serum with a somatic antibody titre of < 4, it was necessary to absorb in several successive stages with the growth from about 100 plates of nutrient agar. Intact living bacilli were used for the early stages (e.g. first four absorptions) and bacilli disrupted by boiling at 100° C. for 1 hr. for the later (e.g. last two absorptions). This procedure would remove antibodies for all superficial somatic antigens and thermostable intracellular antigens. After absorption, each fimbrial antiserum was tested against the homologous fimbriate-phase bacilli and was found to agglutinate these in the same manner and to the same high titre as before the absorption. It was thus shown that fimbrial antibody is responsible for the rapid, loosely floccular agglutination of fimbriate bacilli. The failure of non-fimbriate-phase bacilli to absorb fimbrial antibody proves that such bacilli do not contain any fimbrial antigen, either superficially or intracellularly (the agglutinin-binding property of fimbrial antigen is not destroyed by the boiling treatment used to disrupt the absorbing non-fimbriate bacilli; see below).

Pure fimbrial antiserum strongly inhibited the haemagglutinating activity of fimbriate *Sh. flexneri*. When an equal volume of undiluted serum was added to the red-cell suspension before addition of the bacilli, the resulting haemagglutination reaction was greatly diminished (reaction negative or minimal, ' + ') as compared with the strong reaction (' + + + ') given in a control test using saline in the place of serum. Antiserum for non-fimbriate-phase bacilli gave no inhibition of haemagglutination when tested similarly. The haemagglutination-inhibiting power of fimbrial antiserum was directly related to its antifimbrial bacterial agglutinin titre; it diminished with dilution of the serum, becoming inappreciable when the agglutinin titre was under 120.

The combining affinity for fimbriae possessed by the antibodies in fimbrial antiserum, was demonstrated by electron-microscopy. Agglutinated fimbriate bacilli from a 1 hr. old mixture with pure fimbrial antiserum diluted 1 in 120, were fixed

with formaldehyde, washed and examined in shadow-cast films. The adjacent fimbriae of individual bacilli were then seen to adhere closely side by side in bundles of two to six, or more; single fimbriae appeared to be coated and thickened by an irregular deposit which obscured their normally clear-cut outline (Pl. 4, fig. 5). In contrast, a preparation of the same fimbriate bacilli agglutinated by mixture with homologous non-fimbrial antiserum (somatic agglutination), showed normal, sharply outlined fimbriae radiating separately from the bacilli (Pl. 4, fig. 6).

Relationship of the fimbrial antigens in strains of different O-serotypes

Antisera were prepared separately against living fimbriate-phase cultures of five *flexneri* strains representing different O-serotypes: Type 1*a* (NCTC 8192), Type 2*b* (NCTC 8518), Type 3 (Edin. 36), Type 4*a* (Edin. 9) and Type 5 (NCTC 8524). Each crude fimbrial antiserum was then tested for agglutination against fimbriate cultures of each of the five strains. As shown in Table 1, each serum agglutinated all the heterologous serotype cultures to the same high titre as the homologous culture. Similar cross-agglutination tests were made with pure fimbrial antisera. Each of the five crude fimbrial antisera was purified by absorption with the homologous non-fimbriate-phase culture until its agglutinin titre for this was < 4. The fimbrial agglutinin titres were not changed by the absorption; cross-agglutination tests between the purified sera and the five fimbriate-phase cultures gave results identical to those obtained with the crude sera (as in Table 1). This proves that fimbrial agglutinins were responsible for the cross-agglutination reactions.

Table 1. *Agglutinin titres of crude fimbrial antisera for fimbriate cultures of different* flexneri *O-serotypes*

Fimbriate bacillary suspensions of serotypes	Antisera for strains of serotypes				
	1*a*	2*b*	3	4*a*	5
1*a*	7680	7680	15,360	15,360	1920
2*b*	7680	7680	15,360	15,360	1920
3	7680	7680	15,360	15,360	1920
4*a*	7680	3840	15,360	15,360	1920
5	7680	7680	15,360	7,680	1920

Fimbriate cultures of twenty-three further strains in Types 1*a*, 2*a*, 2*b*, 3, 4*a*, 5, *X* and *Y* were tested with one of the pure fimbrial antisera. Every strain was agglutinated to the same titre as the homologous strain, irrespective of the difference in O-serotype. Correspondingly, the sera inhibited the haemagglutinating activity of all strains equally. The universal similarity of fimbrial agglutinin titre suggests that the fimbriae have an identical antigenic composition in all *flexneri* strains and serotypes.

The complete identity of the fimbrial antigens in the different *flexneri* serotypes was confirmed in cross-absorption experiments. Table 2 shows the results of an experiment made with the pure fimbrial antiserum for the O-serotype-3 strain. Five aliquot portions of this serum were absorbed separately with fimbriate

cultures of the strains representing the five serotypes. After similar successive stages of absorption, each aliquot was tested for agglutination against each of the fimbriate cultures. The results showed that the five different O-serotype strains were identical in the agglutinin-binding activity and specificity of their fimbriate phase. Each gave a similar progressive reduction and eventual elimination of all

Table 2. *Agglutinin titres of pure fimbrial antiserum for a* flexneri *O-serotype* 3 *strain tested after cross-absorption with fimbriate cultures of five different O-serotypes*

	Pure fimbrial antiserum									
		After first-stage absorption with fimbriate bacilli of serotypes					After third-stage absorption with fimbriate bacilli of serotypes			
Fimbriate bacillary suspensions of serotypes	Before absorption	1*a*	2*b*	3	4*a*	5	1*a*	2*b*	3	4*a*	5
1*a*	15,360	240	120	120	120	120	<4	<4	<4	<4	<4
2*b*	15,360	120	240	120	240	120	<4	<4	<4	<4	<4
3	15,360	120	120	240	240	120	<4	<4	<4	<4	<4
4*a*	15,360	120	240	240	120	240	<4	<4	<4	<4	<4
5	15,360	240	120	120	240	120	<4	<4	<4	<4	<4

fimbrial agglutinins for the homologous and heterologous serotype strains. Corresponding cross-absorption experiments were made with the pure fimbrial antisera for the Type 1*a*, 2*b*, 4*a* and 5 strains. These yielded results exactly comparable with those obtained for the Type 3 serum. The finally absorbed sera in all cases were found to have lost their haemagglutination-inhibiting activity along with their fimbrial agglutinins.

Thermolability of fimbrial antigens

Heating at 100° C. removed the fimbriae from fimbriate bacilli and so destroyed their haemagglutinating activity, fimbrial serum agglutinability, fimbrial agglutinin-binding activity, fimbrial agglutinogenicity and O-inagglutinability (Table 3).

(*a*) The effect of heat on the *morphology of fimbriate bacilli* was shown by electron-microscopy. The bacilli centrifuged from a 10 ml. broth culture were washed in saline (0·85 % NaCl), resuspended in 0·5 ml. fresh saline and heated for ½, 1 or 2½ hr. at 60, 90, 100 or 120° C. (this last by autoclaving). The suspension was then diluted to about 15 ml. with distilled water and a drop mounted for electron-microscopy without further washing; this showed the bacilli together with any fimbriae detached by the heating ('whole suspension', Table 3). A second preparation was made of the heated bacilli after centrifugation and resuspension in fresh distilled water ('bacillary deposit'); this was cleaner but lacked the detached fimbriae. Similar results were obtained with strains of all *flexneri* serotypes. After heating for ½–1 hr. at 60° C., the bacilli remained fully and normally fimbriate. After ½–1 hr. at 90° C., most bacilli showed loss of all their fimbriae and the remainder loss of all but a few; the bacillary cell-walls showed evidence of disintegration and large numbers of detached, but otherwise normal fimbriae were present in the background of 'whole suspension' preparations (Pl. 3, fig. 3). After

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1 hr. at 100° C., all fimbriae were separated from the bacilli and some appeared altered in form, many elongated filaments being seen (Pl. 3, fig. 4). After $2\frac{1}{2}$ hr. at 100° C. or $\frac{1}{2}$ hr. at 120° C., most fimbriae were apparently disintegrated and those remaining were much altered in morphology.

Table 3. *Effect of heat on the fimbrial serum agglutinability, fimbrial agglutinin-binding properties, haemagglutinating activity and morphology of a fimbriate* Sh. flexneri *Type 4a strain suspended in saline*

Fimbriate bacillary suspensions	Antibody binding: Agglutinin titre of pure antifimbrial serum for		Antibody binding: Agglutinin titre* of pure antifimbrial serum after absorption with		Antibody binding: Haemagglutination† inhibiting power of serum absorbed with		Haemagglutinating power of whole suspension	Electron-microscopical appearance of whole suspension
	Whole suspension	Bacillary deposit	Whole suspension	Bacillary deposit	Whole suspension	Bacillary deposit		
Unheated	122,880	122,880	480	480	–	–	+++	Normal fimbriae on most bacilli
60° C. (1 hr.)	122,880	122,880	480	480	–	–	+++	
90° C. (1 hr.)	960‡	<30	480	122,880	–	++	–	Fimbriae absent from most bacilli; innumerable detached normal fimbriae
100° C. (1 hr.)	480‡	<30	480	122,880	–	++	–	
100° C. ($2\frac{1}{2}$ hr.)	480‡	<30	240	122,880	–	++	–	Fimbriae absent from all bacilli; detached fimbriae disintegrated or altered in form
120° C. ($\frac{1}{2}$ hr.)	<30	<30	240	61,440	–	++	–	

Whole suspension: bacillary suspension uncentrifuged after heating.
Bacillary deposit: fresh saline suspension of bacilli centrifuged from the heated suspension.
* Titres stated have been corrected for twofold dilution during absorption. Both series of absorbed sera were tested against unheated fimbriate bacilli.
† – =no inhibition; ++ =haemagglutination reduced from +++ to +.
‡ No clearing (see text).

(*b*) The effect of heat on the *haemagglutinating activity* of fimbriate bacilli was tested for *flexneri* strains of all serotypes. The bacilli from 10 ml. broth culture were centrifuged and re-suspended in 0·5 ml. saline and heated in a water-bath. After various intervals a drop was removed and mixed with the red cells on a cold tile. As compared with the strong haemagglutination reaction (++++) given by the suspension before heating, the reaction after heating for 1 hr. at 60° C. was undiminished and that after 1 hr. at 70° C. was weakened (++). All haemagglutinating activity was lost after 1 hr. at 80° C., or after 1–2 min. at 90° C., or higher. Since heating at 80–90° C. detaches the fimbriae without disintegrating them, the free fimbriae must be incapable of binding red cells together.

(*c*) The effect of heat on the *fimbrial serum agglutinability* of the bacilli was

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investigated. Fimbriate bacilli of a *flexneri* Type 4*a* strain were centrifuged from broth cultures, suspended in saline to about 10^9 bacilli per ml. and heated in a water-bath or autoclave. Agglutination tests with homologous pure fimbrial antiserum were made on two equal portions of the heated suspension: (1) uncentrifuged 'whole suspension', containing the bacilli together with any detached fimbriae, and (2) a suspension in fresh saline of bacilli centrifuged from the heated suspension; this 'bacillary deposit' lacked the detached fimbriae since these did not sediment at 3000 r.p.m. The results are shown in Table 3, columns 1 and 2. After heating for 1 hr. at 60° C., both the 'whole suspension' and the 'bacillary deposit' showed normal susceptibility to agglutination, reacting at the same high dilution (122,880) as before heating. This accords with the electron-microscopic finding that fimbriae are not lost at 60° C. After 1 hr. at 90° C., or higher, the 'bacillary deposit' showed complete loss of agglutinability, failing to react even with the lowest serum dilution (30); this was presumably due to the removal of fimbriae. In contrast, after heating at 90° C. or 100° C., the 'whole suspension' (containing the detached fimbriae) remained susceptible to an atypical agglutination or precipitation reaction with low serum dilutions (e.g. titre 480). This reaction differed from the agglutination of unheated bacilli in that the flakes were very fine and remained in suspension. Since the precipitable material was absent from the 'bacillary deposit' of the heated suspension, it presumably consisted of the detached fimbriae. In accordance with this it was found that the cell-free supernatant obtained on centrifuging a boiled bacillary suspension gave a definite precipitin reaction with low dilutions of the pure fimbrial antiserum. Autoclaving at 120° C. destroyed the serum precipitability of 'whole suspensions'.

(*d*) The effect of heat on the *antibody-binding capacity* of fimbriae was investigated. Fimbriate *flexneri* Type 4*a* bacilli were centrifuged from 10 ml. broth, suspended in 1 ml. saline to a density of about 2×10^{10} bacilli per ml. and then subjected to heating. Two 0·5 ml. amounts of homologous pure fimbrial antiserum were separately absorbed for 2 hr. at 37° C. with two equal portions of the heated suspension: (1) 0·5 ml. uncentrifuged 'whole suspension', containing bacilli and detached fimbriae, and (2) 0·5 ml. fresh saline suspension of the 'bacillary deposit' centrifuged from 0·5 ml. heated suspension. The serum, now diluted 1 in 2, was separated from the absorbing bacilli by centrifugation and examined for residual agglutinins and haemagglutination-inhibiting antibodies in tests with unheated fimbriate bacilli. The results are shown in Table 3, columns 3–6. Antibody-binding activity was unaffected by heating for 1 hr. at 60° C.; the heated bacilli reduced the fimbrial agglutinin titre as much as unheated bacilli (from 122,880 to 480). After heating for 1 hr. at 90° C., or higher, the 'bacillary deposits' of the heated suspensions showed complete loss of binding activity for fimbrial agglutinins and haemagglutination-inhibiting antibodies; this was presumably due to the loss of fimbriae. In contrast, the uncentrifuged 'whole suspensions' retained full antibody-binding activity in spite of heating for 1–2½ hr. at 100° C. or for ½ hr. at 120° C.; the retained activity is ascribed to the heat-detached fimbriae which were shown by electron-microscopy to be altered in form or disintegrated by such heating.

(*e*) The *agglutinogenicity* of fimbriae was destroyed by heat more readily than the agglutinin-binding activity. Whole suspensions of *flexneri* cultures heated for 2½ hr. at 100° C. did not stimulate production of fimbrial antibodies when injected into rabbits. The final fimbrial agglutinin titre, determined with fimbriate bacilli of a heterologous O-serotype, was no higher than the pre-immunization titre (60).

Effect of chemical reagents

The presence of 0·25 % formaldehyde had no effect on the morphology, haemagglutinating activity or serum agglutinability of fimbriate *Sh. flexneri*. Treatment with 50 % ethyl alcohol at 37° C. for 20 hr., or with 95 % ethyl alcohol at 45° C. for ½ hr., diminished but did not abolish the agglutinability of fimbriate bacilli by pure fimbrial antiserum (e.g. titre reduced 2–8-fold); the alcoholized bacilli showed weakened haemagglutinating activity and, in electron-micrographs, shortened and scantier fimbriae. Treatment with 0·005 N-HCl for 5 min. at 37° C., Duncan's (1935) method for destroying flagellar antigens, did not affect the haemagglutinating activity and fimbrial serum agglutinability of the bacilli. Treatment with N-HCl for 20 hr. at 37° C. removed the fimbriae and destroyed these properties.

Relationships with fimbrial antigens in other genera

Cross-agglutination tests showed that the fimbrial antigens of *Sh. flexneri* were shared in minor part by the majority of fimbriate *Bact. coli* strains. Pure fimbrial antiserum for a *flexneri* Type 2*b* strain (NCTC 8518) was tested against fimbriate cultures of the homologous strain, a *flexneri* Type 4*a* strain (Edin. 9) and the twenty-six non-haemolytic fimbriate *coli* strains of Duguid *et al.* (1955); the agglutinin titres are shown in Table 4, column 1. The *flexneri* serum gave typical fimbrial agglutination with all the *coli* strains, but at much lower titres (e.g. 120–480) than for the *flexneri* strains (titre 7680). This suggested that the fimbriae of *Sh. flexneri* contain a major *flexneri*-specific antigen not present in *coli* strains, and one or more minor *flexneri-coli* shared antigens present in most *coli* strains.

The same series of strains were tested against three pure fimbrial antisera for *coli* strains nos. 93, 108 and 208 (Table 4, columns 2–4). These had been prepared by injection of the fimbriate *coli* culture and absorbed with the homologous non-fimbriate-phase culture, living and boiled. Each *coli* serum was found to agglutinate a few, apparently related *coli* strains to the same high titre (e.g. 1920) as the homologous strain, and all other *coli* strains, as well as the *flexneri* strains, to a lower titre (e.g. 240). This suggested that the fimbriae of *Bact. coli* contain a major *coli* type-specific antigen shared only within groups of related strains (e.g. the 93, 108 and 208 groups, Table 4), as well as the minor *flexneri-coli* shared antigens. Similar tests were made with forty haemolytic fimbriate *coli* strains; the results resembled those for the non-haemolytic strains, except that six haemolytic strains were not agglutinated by any of the four sera and thus presumably lacked the *flexneri-coli* shared antigens. Flagellar agglutinins were not involved in the reactions of the *coli* sera, since strain 93 was non-flagellate and flagellar agglutinins for the poorly flagellate strains 108 and 208 would be removed from the sera by

Table 4. *Agglutinin titres of pure fimbrial antisera for a* Sh. flexneri *Type 2b and* Bact. coli *strains nos.* 93, 108 *and* 208, *before and after absorption with a fimbriate* Sh. flexneri *Type 4a*

(Sera tested against fimbriate cultures of two *flexneri* and twenty-six non-haemolytic *coli* strains.)

	Pure fimbrial antisera						
		coli sera unabsorbed with fimbriate *flexneri*			**coli* sera absorbed with fimbriate *flexneri*		
Fimbriate bacillary suspensions	*flexneri* 2*b*	93	108	208	93	108	208
flexneri Type 2*b*	7680	480	120	120	<30	<30	<30
flexneri Type 4*a*	7680	960	240	240	<30	<30	<30
coli strains							
no. 93	240	3840	120	240	3840	<30	<30
56	480	1920	120	240	1920	60	<30
103	240	1920	60	480	1920	<30	<30
128	240	3840	240	480	1920	<30	<30
207	240	3840	120	480	1920	<30	<30
228	480	1920	120	480	960	<30	<30
108	120	240	3840	120	<30	3840	30
58	120	120	960	240	<30	960	<30
99	120	240	1920	120	<30	1920	<30
106	120	120	960	240	<30	1920	<30
109	60	240	3840	480	<30	3840	<30
111	120	120	1920	240	<30	1920	<30
208	120	240	120	1920	30	<30	1920
51	240	120	120	960	60	60	480
102	240	240	120	480	<30	30	480
112	240	120	240	960	<30	30	240
116	240	120	120	480	<30	<30	480
122	240	120	120	480	<30	30	480
53	240	120	240	480	<30	<30	<30
54	120	120	240	240	<30	60	<30
97	240	120	<30	<30	<30	<30	<30
117	240	240	480	240	<30	60	<30
195	240	120	240	240	<30	60	<30
201	240	120	120	240	<30	<30	<30
253	960	960	120	240	<30	<30	<30
286	60	120	240	240	<30	<30	<30

* Residual high titres are due to antibodies for *coli* type-specific fimbrial antigens; the low titre antibodies for minor *flexneri-coli* shared fimbrial antigens have been removed by the absorption.

the absorption with non-fimbriate-phase bacilli. Furthermore, many parallel tests were made with bacilli deflagellated by 0·005 N-HCl and these were agglutinated to much the same titre as the untreated bacilli.

Cross-absorption tests confirmed the existence of the type-specific and shared fimbrial antigens. The pure fimbrial antisera for *coli* strains 93, 108 and 208 were each absorbed with a fimbriate culture of *Sh. flexneri* Type 4*a* (Edin. 9) and then tested for residual fimbrial agglutinins (Table 4, columns 5–7). It was found that

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the *flexneri* absorption in no case reduced the high titre agglutination given by each *coli* serum with its homologous strain and closely related strains. This showed that the Flexner bacilli did not contain any of the three major *coli* type-specific fimbrial antigens. On the other hand, the absorption in every case virtually eliminated the low-titre cross-agglutination for the less closely related strains. This confirms the existence of minor *flexneri-coli* shared antigens in all twenty-six non-haemolytic *coli* strains.

Table 5. *Agglutinin titres of* Sh. flexneri *Type* 4*a fimbrial antiserum for fimbriate cultures of a* flexneri *Type* 4*a strain and* Bact. coli *strains nos.* 93, 108, 208, 51 *and* 253, *before and after absorption, first with non-fimbriate and secondly with fimbriate cultures*

	flexneri Type 4*a* fimbrial antiserum						
		Absorbed with non-fimbriate 4*a*, 51, 93, 108 and 208 bacilli*	Separate aliquots secondarily absorbed with fimbriate cultures of *coli* strains				
Fimbriate bacillary suspensions	Crude antiserum		93	108	208	51	253
4*a*	122,880	122,880	122,880	122,880	122,880	122,880	< 30
93	960	480	< 4	120	60	60	< 30
108	480	480	120	< 4	120	< 30	< 30
208	120	120	60	120	< 4	120	< 30
51	7,680	7,680	3,840	3,840	3,840	< 4	< 30
253	30,720	15,360	7,680	7,680	15,360	7,680	< 30

* In tests with non-fimbriate-phase cultures of Type 4*a*, strains 93, 108 and 208, and boiled cultures of strains 51 and 253, the crude antiserum showed somatic agglutination titres of, respectively, 1920, < 30, 60, < 30, < 30 and < 30. After absorption with the non-fimbriate bacilli, no agglutination was given even at a 1 in 4 dilution.

The converse absorption tests were made with fimbrial antiserum for the *flexneri* Type 4*a* strain. Agglutinin titres were determined for the homologous strain and all the non-haemolytic *coli* strains except nos. 97 and 286; representative results are shown in Table 5. The crude antiserum was purified by absorption with living and boiled non-fimbriate-phase cultures of the 4*a* strain and *coli* strains 93, 108 and 208, and with a boiled culture of *coli* strain 51, which never occurs in a purely non-fimbriate phase. As shown in the first two columns of Table 5, this non-fimbrial absorption did not reduce appreciably the specific or cross-reacting fimbrial agglutinins, although it removed all antibodies for the somatic antigens (Table 5, footnote). Following the non-fimbrial absorption, five aliquot portions of the *flexneri* serum were separately absorbed with fimbriate cultures of *coli* strains 93, 108, 208, 51 and 253, respectively, and were then tested for residual agglutinins against the *flexneri* and twenty-four *coli* strains (e.g. columns 3–7, Table 5). The majority (fifteen) of the *coli* strains gave results similar to those shown for the 93, 108 and 208 suspensions. The *flexneri* serum, before fimbrial absorption, agglutinated them to low titres (120–960). Fimbrial absorption with any of the five *coli* strains reduced these cross-reacting agglutinins to an even lower titre (120 or less). Presumably this was due to removal of antibodies for minor *flexneri-coli* shared

antigens occurring in all the strains. The residual low-titre reactions (60 and 120) possibly were due to sharing of further minor antigens between *Sh. flexneri* and individual *coli* strains.

A greater sharing of fimbrial antigens with *Sh. flexneri* was shown by the *coli* strains 51, 54, 102, 112, 116, 117, 122 and 195 (the '51 group'), and a very close relationship by strain 253. The *flexneri* pure fimbrial antiserum agglutinated these to relatively high titres (1920–7680 for the 51 group), which were not appreciably reduced after absorption with strains 93, 108 and 208. Absorption with strain 51 reduced to a low level (< 30) the titres for all strains of the 51 group, presumably by removing antibodies for a fimbrial antigen shared only by these strains, strain 253 and *Sh. flexneri*. Absorption with strain 51 did not reduce the titre of the *flexneri* serum for the homologous *flexneri* strain (122,880) nor, appreciably, that for strain 253 (15,360), but reduced to a low level (< 240) the titres for all the other *coli* strains. This shows that the major *flexneri*-specific fimbrial antigen was absent from strain 51 and all the other *coli* strains except no. 253. Absorption of the *flexneri* serum with strain 253 removed all fimbrial agglutinins for the homologous *flexneri* strain and the twenty-four *coli* strains (to titres < 30). Thus, alone of the *coli* strains, no. 253 shared all the fimbrial antigens of *Sh. flexneri*; it was a typical faecal strain, motile, lactose fermenting and indole producing.

We did not find sharing of the *flexneri* antigens by *Salmonella*, *Bact. cloacae* or *Proteus*. The *flexneri* Type 4*a* pure fimbrial antiserum did not agglutinate fimbriate cultures of *Salmonella typhi*, *S. paratyphi* B, *S. enteritidis*, *S. typhimurium* and *S. thompson*, nor those of eleven *Bact. cloacae* strains (titres < 10). It did not inhibit haemagglutination by fimbriate cultures of thirty-two strains of *Proteus vulgaris* and *Pr. mirabilis* (in their fimbriate phase these various *Proteus* strains were constantly autoagglutinable and unsuitable for serum agglutination tests). Inhibition was to be expected if *Proteus* shared even a minor fimbrial antigen with *flexneri*, since it had been found that a *flexneri* serum inhibited haemagglutination by *coli* strain 93 and the *coli*-93 serum inhibited haemagglutination by *flexneri*.

Occurrence of fimbrial antibodies in human sera

We examined eighty-one human sera mostly submitted for routine antenatal Wassermann tests. All but one gave typical fimbrial agglutination of a fimbriate *flexneri* culture, at titres ranging from 30 to 1920 (mostly about 240). Twelve were absorbed with a non-fimbriate-phase culture of the same *flexneri* strain and on retesting showed no reduction in titre for the fimbriate culture. A second absorption with the fimbriate bacilli in all cases reduced the agglutinin titre to < 30. The main natural agglutinins of human sera detectable by tests with fimbriate cultures were thus shown to be fimbrial antibodies. In parallel tests made with forty-nine of the sera, all except five agglutinated a fimbriate *Bact. coli* culture (strain 93, 99 or 102) to virtually the same titre as the *flexneri* culture (difference not greater than two-fold). It thus seems probable that the *flexneri* fimbrial agglutinins in most human sera are directed to the minor *flexneri-coli* shared fimbrial antigens and are formed as a result of stimulus by the intestinal *Bact. coli*.

Forty-eight of the sera were tested for haemagglutination inhibition against the same fimbriate *flexneri* culture as used for the agglutination test. Since these sera commonly contained natural haemagglutinins for guinea-pig red cells, but rarely any for fowl cells, the latter were used for the inhibition tests and the sera initially absorbed with such cells to eliminate haemagglutinins. The sera were found to inhibit fimbrial haemagglutination to an extent roughly corresponding with their fimbrial agglutinin titre. Thus, sera of titre < 120 gave little or no inhibition, while those of titre 960–1920 were strongly inhibitory, reducing the reaction from + + + to +. The whole of the haemagglutination-inhibiting activity of the sera was due to absorbable fimbrial antibody, since it was eliminated by absorption with the fimbriate *flexneri*, but unaffected by absorption with the corresponding non-fimbriate-phase culture.

DISCUSSION

Our results show that the fimbriae of *Sh. flexneri* and *Bact. coli* bear important antigens, and that the agglutination of fimbriate bacilli by homologous antiserum is mainly due to the action of fimbrial antibodies. The cross-agglutination and absorption experiments showed that the fimbriae of all strains of *Sh. flexneri*, irrespective of O-serotype, were identical in antigenic composition. They contained a major, *flexneri*-specific antigen, absent from all *Bact. coli* strains except one (no. 253), one or more minor *flexneri-coli* antigens shared with most *coli* strains and at least one further antigen shared with a few *coli* strains (51 group). The *flexneri* antigens apparently were not shared by fimbriate strains of *Salmonella*, *Bact. cloacae* and *Proteus*.

When fimbriate bacilli are mixed with a crude homologous antiserum, fimbrial agglutination is dominant and prevents observation of somatic agglutination involving the O-antigen; it occurs more rapidly than the latter, to a higher titre and without specificity corresponding to the O-serotype. Thus, when attempting to identify the O-serotype, it is necessary to avoid fimbrial agglutination. Non-fimbriate bacillary suspensions should be used, either (1) non-fimbriate-phase cultures selected by serial aerobic cultivation on well-dried agar plates, or (2) bacilli defimbriated by heating at 100° C. and centrifuged from the detached fimbriae. When using unboiled, fimbriate-phase broth cultures, we found it impossible to determine the O-serotype of *flexneri* strains by tube-agglutination tests with type-specific diagnostic sera (kindly provided by Dr Patricia Carpenter of the Dysentery Reference Laboratory, Colindale); some of these sera gave strong fimbrial agglutination of strains of heterologous O-serotypes. In normal diagnostic practice, however, the procedures of isolation and subcultivation on agar prior to serological testing, usually ensure that *Sh. flexneri* is in the non-fimbriate phase. Typing sera free from immune and natural fimbrial antibodies could be prepared by absorption with a fimbriate *flexneri* culture of heterologous O-serotype.

The rapid, loosely floccular nature of fimbrial agglutination makes liable its confusion with flagellar agglutination in species such as *Bact. coli*. It may be distinguished from the latter by its persistence after treatment of the bacilli with 0·005 N-HCl or 50 % ethyl alcohol. In being thermolabile, superficial and liable to

confer relative O-inagglutinability, fimbrial antigens resemble the K-antigens (L, A and B) which occur in many strains of *Bact. coli* (Kauffmann, 1947). K-antigens have been found only rarely in *Sh. flexneri* (Edwards & Ewing, 1955). However, the fimbrial antigens of this species would seldom be encountered by serologists using agar cultures or first broth subcultures from agar, since these are normally non-fimbriate. On the other hand, many *Bact. coli* strains remain partly fimbriate on agar (Duguid *et al.* 1955) so that their fimbrial antigens may have been observed.

In spite of this general resemblance, we were unable to identify fimbrial antigens with any of the previously described K-antigens. Fimbrial antigens differ from the L-antigens of Kauffmann (1943) in that the L-agglutinability of bacilli is destroyed by heating at 60° C. for 1 hr. or by exposure to 50% ethyl alcohol for 20 hr. at 37° C., while fimbrial agglutinability is unaffected by heating at 60° C. and only slightly reduced by alcohol treatment. Fimbrial antigens differ from the capsular A-antigens of Kauffmann (1947) in being thermolabile at 100° C. and in occurring commonly in non-capsulate bacilli (e.g. *Sh. flexneri*). They also differ from the B-antigens of Knipschildt (1946), shown to be capsular by Ørskov (1956), since binding power for fimbrial agglutinins is lost, and that for B-agglutinins retained, in bacilli heated at 100° C. for 1–2½ hr., centrifuged and washed. Moreover, *coli* strains with apparently identical fimbrial antigens possess presumably different B-antigens; e.g. strain 128 (O128, B12) and strain 207 (O111, B4).

The fimbrial antigens bear important resemblances to some of the known cross-reacting antigens of enterobacteriaceae, in particular the X-antigen described in salmonellae by Topley & Ayrton (1924), Happold (1928, 1929) and Cruickshank (1939), the α-antigen of Stamp & Stone (1944) and the β-antigen of Mushin (1949). The α-antigen, however, differs in not reacting with natural agglutinins in normal human sera, while the β-antigen occurs in some species never yet found fimbriate (e.g. *Sh. boydii* Type 6) and rarely in genera which are predominantly fimbriate (e.g. *Salmonella*). The X-antigens resemble fimbrial antigens very closely: in their thermolability, in giving rapid, loosely floccular agglutination, in the cultural conditions favouring their appearance and disappearance, and in occurring as precipitinogens in the supernatants of cultures heated at 100° C. Further serological studies on the fimbrial antigens of salmonellae should reveal whether these are identical with the X-antigens.

SUMMARY

1. 'Pure fimbrial antisera' were prepared for *Sh. flexneri* strains of O-serotypes 1*a*, 2*b*, 3 and 4*a* and 5, by injecting rabbits with a living fimbriate-phase culture and absorbing the crude immune serum with a non-fimbriate-phase culture of the same strain to remove antibodies for the non-fimbrial (somatic) antigens. These sera gave at high titre a rapid, loosely floccular agglutination of all fimbriate-phase *flexneri* cultures, caused adhesion of their fimbriae visible by electron-microscopy and inhibited their haemagglutinating activity. 'Non-fimbrial antisera', prepared by injection of non-fimbriate-phase cultures, were devoid of these activities; they gave somatic-type agglutination with fimbriate bacilli at lower titres than with homo-

logous non-fimbriate bacilli, the fimbriae tending to mask the O-antigens and confer relative O-inagglutinability.

2. Heating at 90° C. detached the fimbriae from the bacilli, so that these lost their haemagglutinating activity, fimbrial serum agglutinability and fimbrial agglutinin-binding power. When heated for 2½ hr. at 100° C., the detached fimbriae retained agglutinin-binding power, but lost their immunogenicity.

3. Cross-agglutination and absorption tests showed that the antigenic composition of the fimbriae was identical in all *flexneri* strains, irrespective of O-serotype. The *flexneri* pure fimbrial antisera agglutinated at low titre sixty out of sixty-six fimbriate *Bact. coli* strains. Absorption tests with a *flexneri* and three *coli* pure fimbrial antisera showed that the fimbriae of *Sh. flexneri* contained a major '*flexneri*-specific antigen', found in only one *coli* strain, one or more minor '*flexneri-coli* antigens' shared by most *coli* strains, and at least one further antigen shared by a few *coli* strains. The *coli* fimbriae also contained a major, '*coli* type-specific antigen' shared in groups of several related strains. The *flexneri* sera did not react with fimbriate strains of *Bact. cloacae*, *Salmonella* and *Proteus*.

4. Fimbrial agglutinins for *Sh. flexneri* were found in eighty of eighty-one normal human sera at titres from 30 to 1920, and in some pre-immunization rabbit sera at titres of 30 to 60.

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EXPLANATION OF PLATES

PLATE 2

Fig. 1. Bacillus from fimbriate-phase broth culture of *Sh. flexneri* serotype 1*a*, in electron micrograph of gold-palladium shadowed film. × 31,000.

Fig. 2. Haemagglutination tile tests after mixing for 5 min. Reactions from left to right are + + + +, + + + and −. × 1¼.

PLATE 3

Fig. 3. Fimbriate *Sh. flexneri* Type 2*a* suspension heated at 90° C. for 30 min. Bacillary cell-wall is disintegrated. The fimbriae have almost all been detached from the bacilli; apart from some fragmentation into short lengths, they are unaltered in appearance. × 22,000.

Fig. 4. Fimbriate *Sh. flexneri* Type 2*a* suspension heated at 100° C. for 1 hr. Bacillary cell-walls are disintegrated. The fimbriae have been detached from the bacilli and some altered, apparently increased in length. × 22,000.

PLATE 4

Fig. 5. Fimbriate *Sh. flexneri* Type 1*a* agglutinated for 1 hr. by a 1/120 dilution of homologous antifimbrial serum. Adjacent fimbriae are mostly adherent to one another in bundles of two to six. Single fimbriae bear an irregular deposit along their edges. × 27,000.

Fig. 6. Fimbriate *Sh. flexneri* Type 1*a* agglutinated for 1 hr. by a 1/60 dilution of antiserum for the non-fimbriate phase of the same strain. The fimbriae radiate freely from the bacilli without adhering to one another and show no irregular deposit on their edges. × 27,000.

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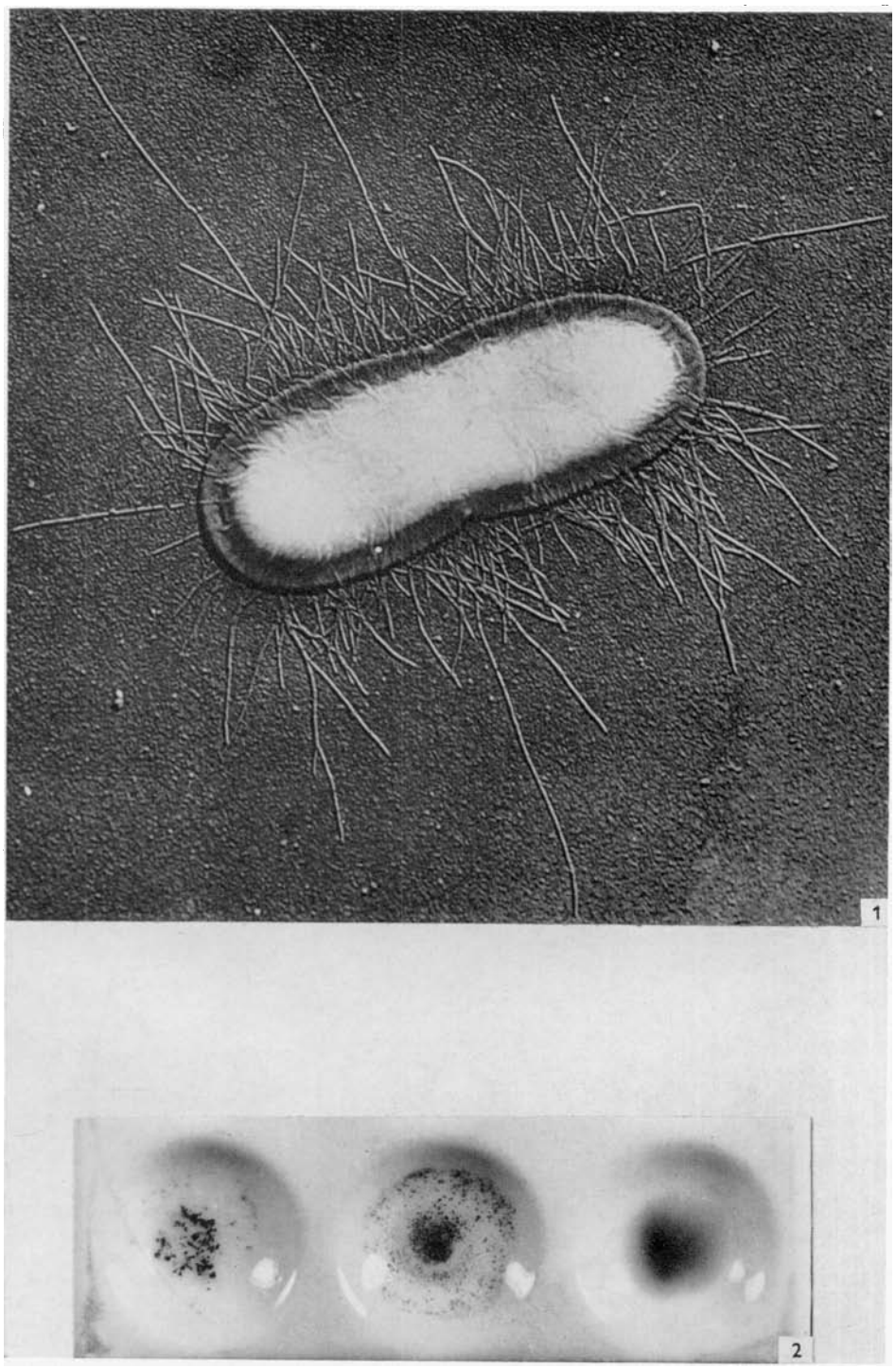

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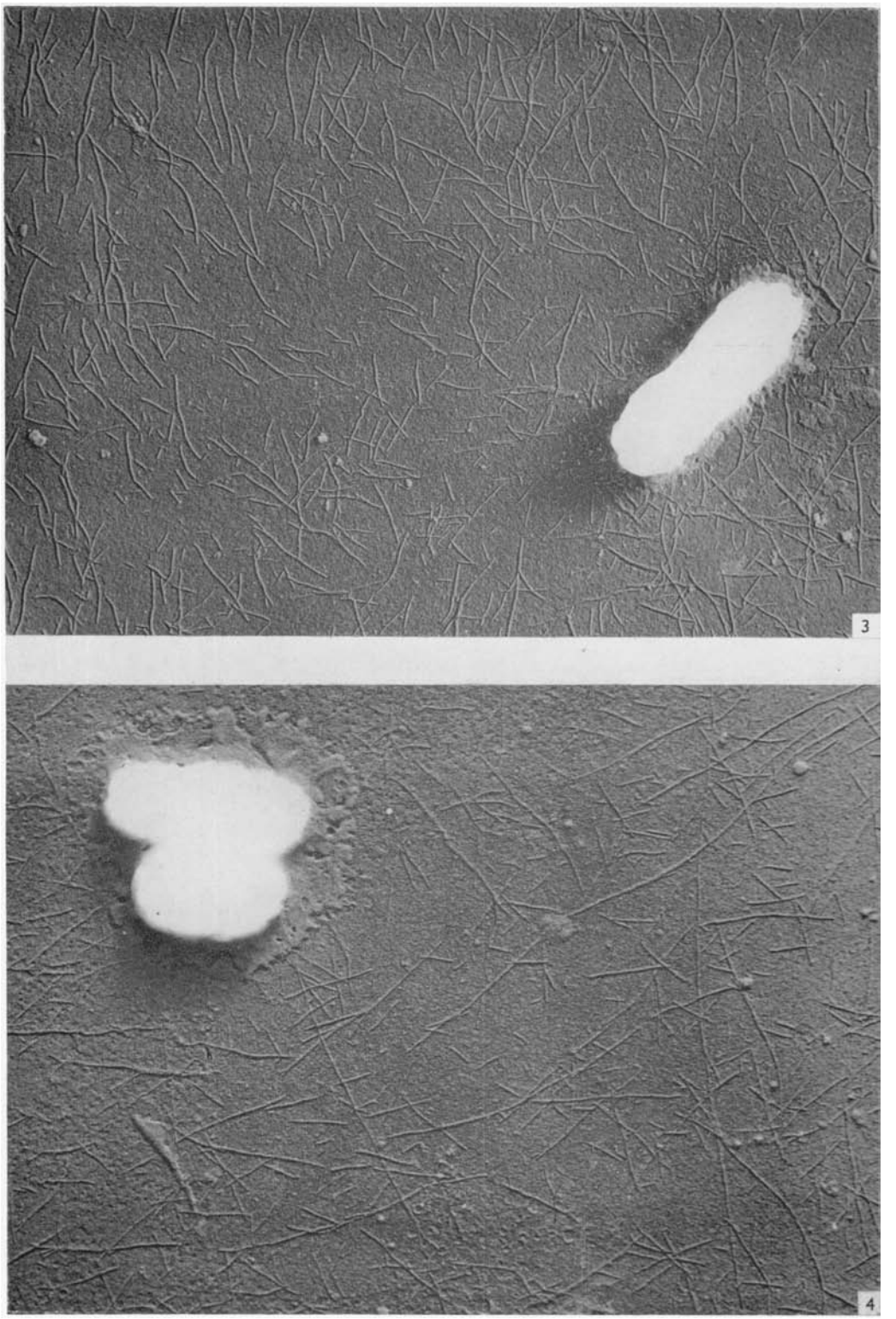

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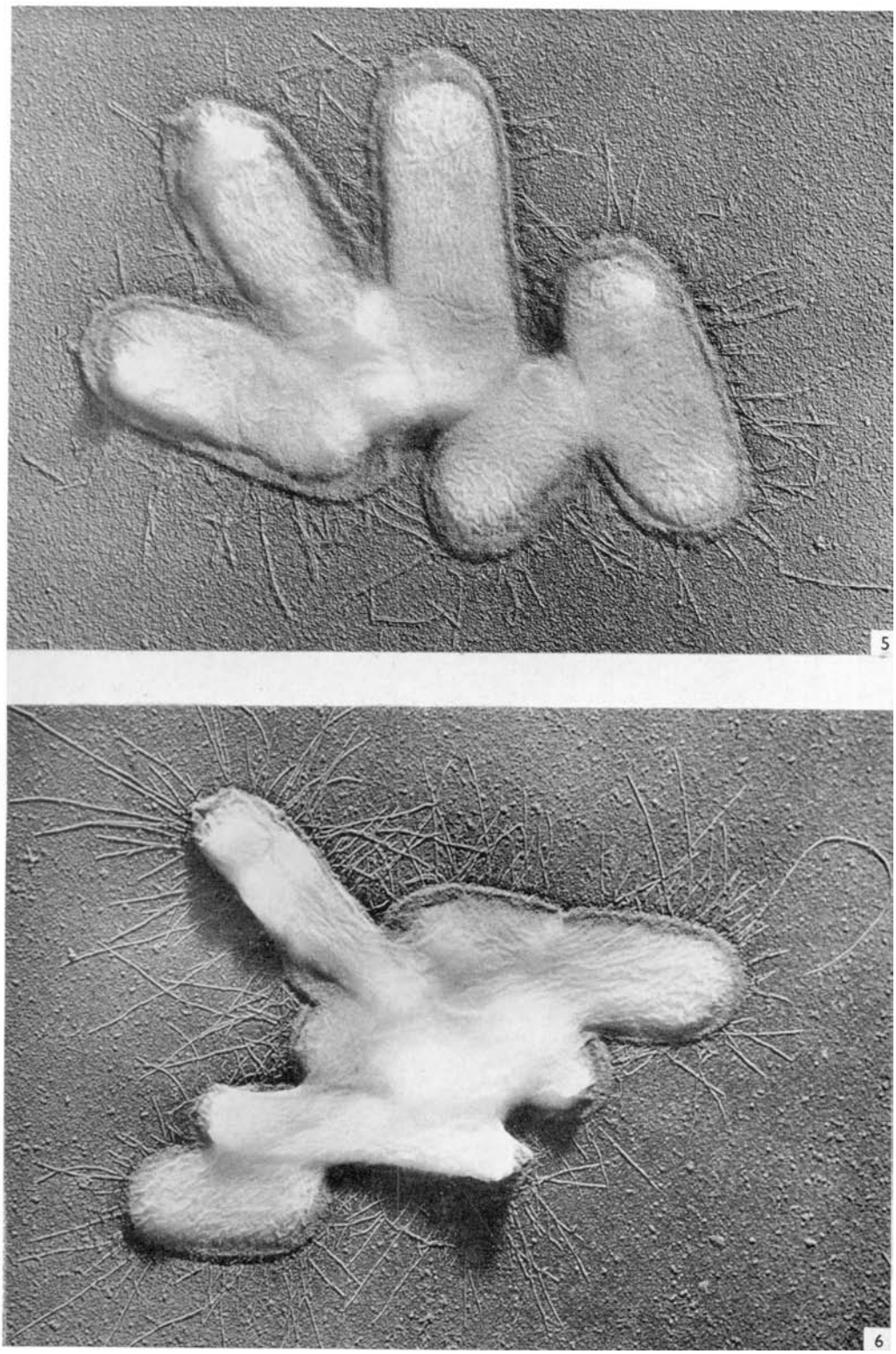

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