CHAPTER III

RESULTS

1. Haemagglutination Properties

1.1 HP and HA with GPE

The ability of *S.typhimurium* F885 to agglutinate GPE was compared with other fimbriate and non-fimbriate strains (table 3). We found that *S.typhimurium* F885 was the most strongly haemagglutinating (HP= 3200), whereas *S. typhimurium* C5, *S.strasbourg* and *S.enteritidis* 11RX gave HP= 800, 1600 and 200 respectively. Haemagglutinating activity was absent from *S.typhimurium* M206 and *E.coli* F492.

The presence and absence of type-1 fimbriae on strain F885 and F492 were confirmed by electron microscope examination, respectively (Fig.4,5)

1.2 Activity of Different Carbohydrates in HAI

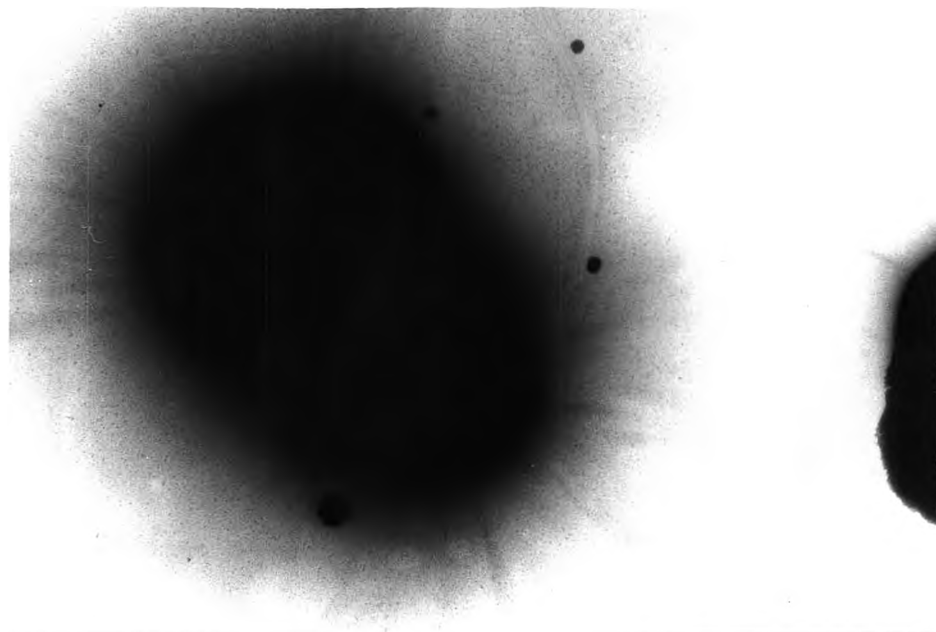
Table 4 gives for various carbohydrates the MIC required to prevent the agglutination of GPE by *S. typhimurium* F885. D-mannose and methyl α-D-mannopyranoside were very effective inhibitors, D-glucose gave a less

Table 3 HP and HA of GPE by various fimbriate and non-fimbriate stains[a]

Strain	HA titer	MHD (cells/ml)	HP
S.typhimurium F885	1:32	$3.12x10^7$	3200
S.typhimurium C5	1:8	$1.25x10^8$	800
S.strasbourg	1:16	$6.25x10^7$	1600
S.enteritidis 11RX	1:2	$5.00x10^8$	200
S.typhimurium M206	N	-	-
E.coli F492	N	-	-

a = Bacterial concentration was $1x10^9$ cells/ml

N = No agglutination



<u>Fig.4</u> Electron micrograph of strain F885 after growth for 48 h in static BHI. x 40,000

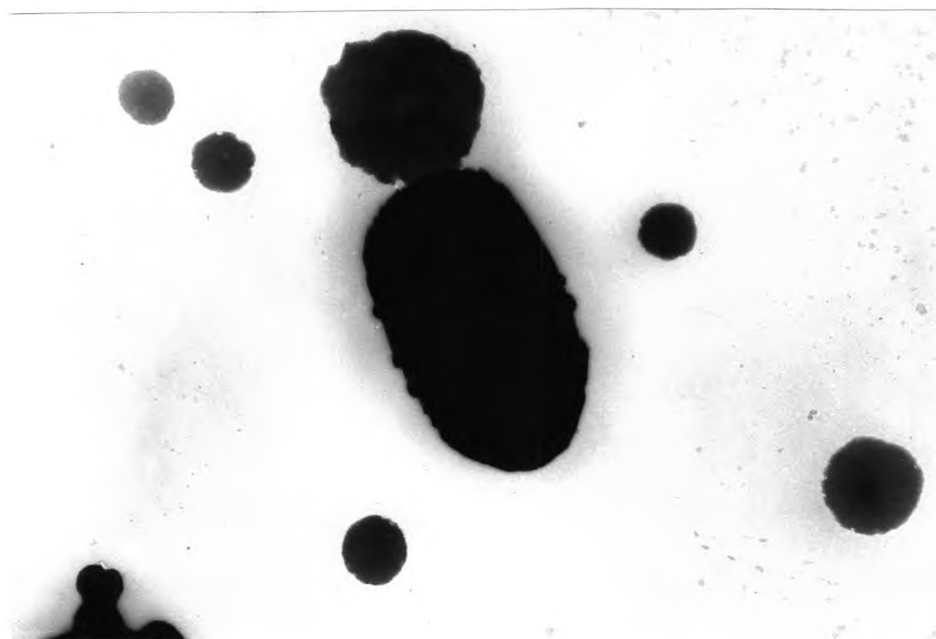


Fig.5 Electron micrograph of strain F492 after growth for 48 h in static BHI. x 30,000

Table 4 Activity of different carbohydrates in HAI of *S.typhimurium* F885[a]

Carbohydrate	MIC (ug/ml)
D-Mannose	24.00 (±0.19)[b]
Methyl α-D-Mannopyranoside	16.97 (±0.16)
D-Glucose	1984.25 (±0.15)
D-Galactose	>5000
D-Altrose	>5000
L-Rhamnose	>5000
Lactose	>5000
Sucrose	>5000

a = Bacterial concentration was four times the MHD

b = Geomatric mean (±SD) of six determinations

inhibition, D-galactose, D-altrose, L-rhamnose, lactose and sucrose had no effect on the agglutination.

2. Purification of Type-1 Fimbriae

2.1 Comparison of Purification Procedures

As shown in table 5, the best method for fimbrial preparation was that of Dodd and Eisenstein. The average yield by this procedure was about 4 mg fimbriae from 20 g (wet weight) of bacteria, whereas the methods of Salit and Gotschlich, and Knutton et al. gave poorer yields of 0.8 and 2.4 mg fimbriae respectively, before spining in a self-generating isopycnic cesium chloride gradient, and gave very low yields of about 0.1-1.0 mg fimbriae of after such treatment.

2.2 According to the Method of Dodd and Eisenstein

After growth for 48 h in static, aerobic BHI broth, the *S.typhimurium* F885 formed pellicles on the culture surface and showed strong HA (table 3). The cells (wet weight = 17 g) from 4 liters of medium were harvested by centrifugation. Fimbriae were detached from bacterial cells using an homogenizer. Pellets of semipure fimbriae after ultracentrifugation gave a product containing approximately 31% of the protein in the original extract. Protein analysis showed that type-1 fimbriae accounted for

Table 5 Comparison of type-1 fimbriae from *S.typhimurium* F885 by various purification procedures

According to the method of	wet wt.(g)	fimbriae (mg)	MHC (ug)
Salit & Gotschlich	20	0.77[a] (±0.38)[b]	396.85(±0.17)[c]
Knutton *et al.*	20	2.37[a] (±1.00)	99.21(±0.17)
Dodd & Eisenstein	20	4.00 (±0.20)	4.91(±0.17)

a = Fimbriae before apply to a self-generating isopycnic cesium chloride gradient

b = Arithmetic mean (±SD), c = Geomatric mean (±SD) of triplicate determination from three batches of purified fimbriae

approximately 6% of the semipure fimbrial preparation after treatment with 5 M urea (table 6). All samples containing fimbriae gave heamagglutination. whereas supernatants without fimbriae gave no heamagglutination (table 6).

3. Determination of Fimbrial Purity and MW

3.1 SDS-PAGE

Fig.6 shows the SDS-PAGE analysis of samples from strain F885 and the sequential purification steps, Lane a shows the marked contamination with other proteins after the blending step. The partially cleared supernatant was then subjected to ultracentrifugation (227,000 x g for 2 h) which completely cleared the supernatant of fimbriae (lane b). The clear gelatinous pellets of semipure fimbriae were resuspened in 5 M urea to disaggregate other protein, leaving the urea-resistant fimbriae intact. Pellets of pure fimbriae (lane e) were obtained by ultracentrifugation (200,000 x g for 16h) through a 1 M urea - 1 M sucrose cushion. The supernatants obtained by such treatment contained no fimbriae (table 6).

MW of type-1 fimbriae from *S.typhimurium* F885 were determined by Rf plots, using linear regression of relative mobility versus known MW on semi-logarithmic paper (Fig.3). Type-1 fimbriae had a approximately MW of 19K, and a minor band of 18K. This minor band protein was not abundant in

Table 6 HA and recovery of protein, type-1 fimbriae during purification[a]

Procedure	Product	Vol ml	Protein (mg/ml)	Protein mg	HA	MHC
Homogenization	Crude extract	49.0	4.0	196.0	1:16	250
UC	Supernatant	42.0	3.2	134.4	N	-
	Pellets	4.0	15.0	60.0	1:8[b]	375
Treatment with 5 M urea, UC	Supernatant	42.5	1.3	55.3	N	-
	Pellets	3.0	1.2	3.6	1:256	4

a = According to the method of Dodd & Eisenstein

b = HA titer from 3.0 mg/ml of protein

UC = Ultracentrifugation

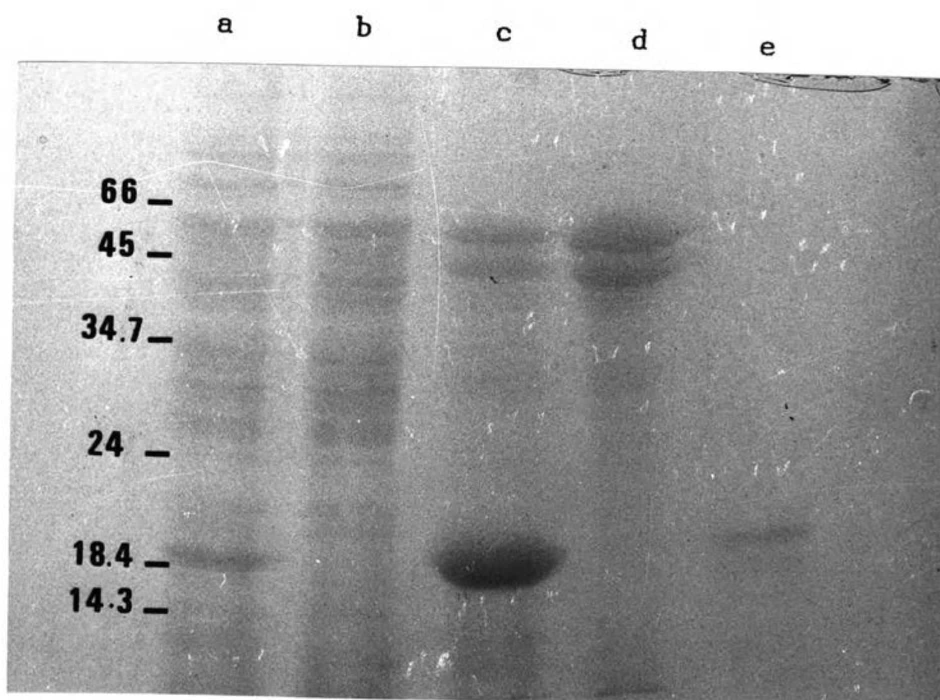


Fig.6 SDS-PAGE analysis of various stages of fimbriae purification from S.typhimurium F885. Lane a, crude extract after homogenization (50 ug); lane b, supernatant after homogenization and ultracentrifugation (40 ug); lane c, pellets of semipure fimbriae (40 ug); lane d, supernatant after treatment with 5 M urea and ultracentrifugation (30 ug); lane e, pellets of pure fimbriae (15 ug); reference MW indicated on the left were as described in the legend to Fig.3.

the starting material (Fig.6a) and seemed to have been enriched in the purification process. Nevertheless, the fimbriae retained their native morphology in the purification process and no contaminating membrane vesicles were seen in electron microscopy (Fig.7).

3.2 IEP

The purity of the type-1 fimbriae preparaton was demonstrated by immunoelectrophoretic analysis of the crude and final Ag preparations. After electrophoresis, rabbit antiserum prepared against the whole cells Ag produced only a single precipitin line with the final Ag preparation, in contrast there were two precipitin lines with this antiserum and the semipure Ag preparation (Fig.8). Also, the native protein exhibited a low electrophoretic mobility. Furthermore, the purified type-1 fimbriae produced a single line of identity when reacted against Ab prepared against the pure Ag (Fig.9,trough A) and against Ab prepared against the whole cells Ag (Fig.9,trough B).

4. Distribution of Live Bacterial Vaccines in Mice After Oral Feeding

Both of two strains examined appered in the Peyer's patches of the small intestine 1 day after mice were fed 1x 10^{10}bacteria (Fig.10). *E.coli* F492 multiplied approximately four fold by day 2, but the numbers then fell ten fold by

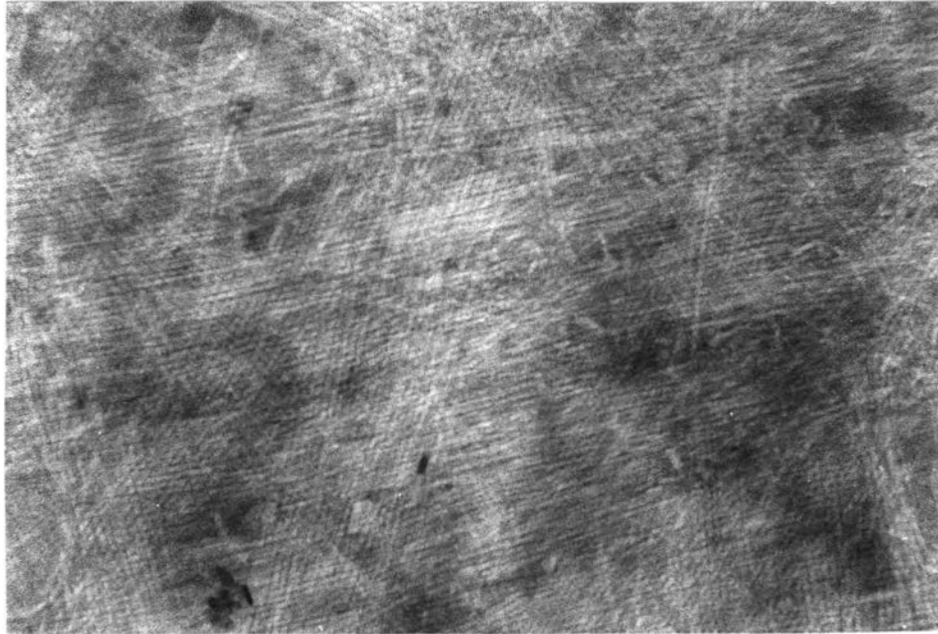


Fig.7 Electron micrograph of purified fimbriae from strain F885. x 118,000

Fig.8 Immunoelectrophoresis of crude Ag preparation in upper well and purified fimbriae Ag in lower well. The trough contained rabbit antiserum prepared against the whole cells.

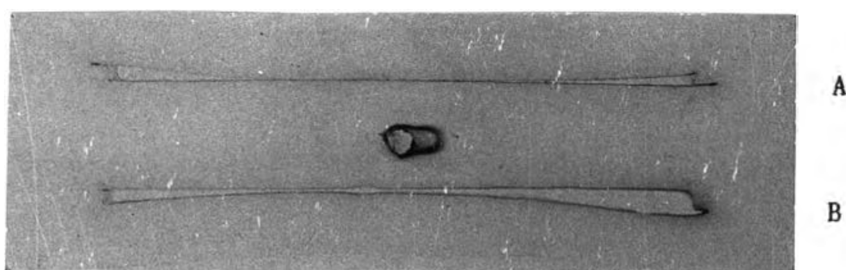


Fig.9 Immunoelectrophoresis of purified fimbriae Ag demonstrating purity. Trough A contains Ab prepared against the purified Ag, and trough B contains Ab prepared against the whole cells.

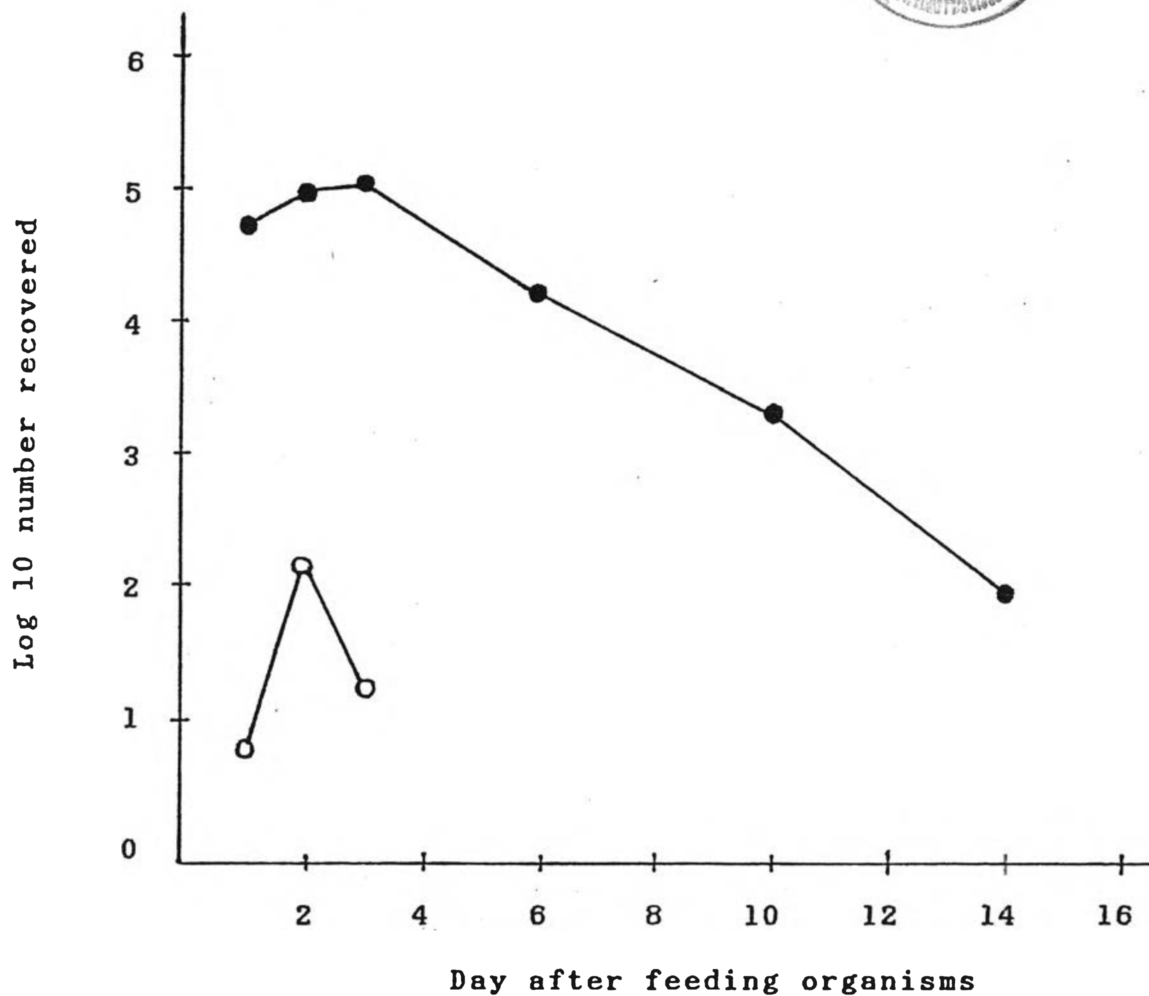


Fig.10 The number of viable bacteria recovered from the Peyer's patches at specified times after oral feeding of BALB/cJ strain mice with 1×10^{10} viable bacteria of S.typhimurium F885 (●) and E.coli F492 (○). Each point represents the mean of numbers recovered from each group of at lest five mice.

day 3 and could no longer be detected at day 4. In contrast S.typhimurium F885 increased in numbers, for at least 3 days, at which point they began to decrease slowly and steadily by about one log every 4 days.

5. LD50 Determination

As shown in table 7, the dilution factor was 10 and 50% end point dilution of the challenge strain C5 was the figure between 10^4 and 10^3 dilution. The % mortality at dilution next below was 25% and the % mortality at dilution next above was 78%. Calculation using the formula described by Reed and Muench (106), showed the LD50 of S. typhimurium C5 when given orally to be 2.96×10^3 organism.

6. Mouse Protection Test

6.1 Protective Immunity Induced by Various Vaccines

As shown in table 8, E.coli F492 used for immunization provided no protection against a challenge with 1×10^6 organisms of S.typhimurium C5 about 1,000 LD50. On the other hand, S.typhimurium F885 and fimbriae from strain F885 were able to confer 80% and 67% protection respectively (fiducial limit <0.005) against a similar challenge.

Table 7 LD50 of the challenge strain C5 in the BALB/cJ mice

Dilution rate	Mortality	Death	Survivor	Total Death	Total Survivor	Percent mortality
10^7	8/8	8	0	32	0	100
10^6	8/8	8	0	24	0	100
10^5	8/8	8	0	16	0	100
10^4	6/8	6	2	8	2	78
10^3	2/8	2	6	2	8	25
10^2	0/8	0	8	0	16	0

Table 8 Resistance of mice against oral challenge with *S.typhimurium* C5 after immunizing with various vaccines

Oral Immunization with 10^{10} organisms of	Survivors / total	Protection		Persistence in Peyer's patches
		% survival over control	Significance of test[a]	
S.typhimurium F885	12/15 (0/15)[b]	80	<0.005	+
E.coli F492	0/15 (0/15)	0	NS	-
Type-1 fimbriae[c]	10/15 (0/15)	67	<0.005	

a = P values were calculated by the Chi-square test

b = Groups of control mice

c = Group of mice were immunized with 50 ug of fimbriae on day 0 (i.p.) and day 12 (s.c.)

NS = Not significant

6.2 Distribution of *S.typhimurium* C5 in Mice After Oral Challenge

As shown in Fig.11, the *E.coli* F492 had little effect on reducing the numbers of organisms recovered compared to the controls, and the subsequent growth both in the Peyer's patches and spleen in these two groups was virtually identical. By day 3, organisms began to appear in the spleen in numbers which rapidly increased over the following 4 days. Death usually occurred between 7 and 9 days.

The level of *in vivo* bacterial growth in control mice, differed from that in the *S.typhimurium* F885 and fimbriae-immunized mice in several respects. Three days after challenge, the number of organisms present in the Peyer's patches and spleen of control mice was many fold higher than in the immune mice. By day 7, the challenge organisms progressively multiplied and reached toxic proportions in control mice, whereas in the immune mice from day 3 to 7 there was only a moderate increase in the number of bacilli. By day 9, all the control mice had succumbed to infection. Conversely, in the *S.typhimurium* F885 and fimbriae-immunized mice, bacterial growth in the Peyer's patches and spleen began to decrease steadily.

Log 10 number recovered

7
6
5
4
3
2
1
0

2 4 6 8 10 12 14 16

Days after challenge

Fig.11 The Number of S.typhimurium C5 recovered from the Peyer's patches (closed symbols), and spleen (open symbols) at specified times after oral infection of mice with $1x10^6$ organisms, normal mice (■,□), mice immunized with $1x10^{10}$ E.coli F492 (▲,△), $1x10^{10}$ S.typhimurium F885 (●,○), 100 ug fimbriae (◆,◇). Each point represents the mean of numbers recovered from each group of at least five mice.