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Interaction of albumin and fusidic acid

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Appendix on interaction of albumin and fusidic acid

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Summary

1. By combining the agar plate diffusion technique for determination of antibiotic activity and zone microelectrophoresis in agar gel, the activity of fusidic acid in individual serum proteins of blood and pus obtained from patients given sodium fusidate revealed albumin to be responsible for the protein binding of this antibiotic.

2. Based on the assumption that only free fusidic acid is microbiologically active, the relationship between the concentration of albumin and the ratio of free to total fusidic acid was determined at four concentrations of free fusidic acid, using as test organisms four differently sensitive variants of a *Staphylococcus aureus* strain. At each concentration an increasing amount of albumin (0–40 mg/ml culture medium) decreased the activity of fusidic acid as determined in serial dilutions (IC50).

3. The law of mass action expressed as Langmuir's adsorption isotherm was valid if a correction for the influence of albumin on the sensitivity of the strain of *Staph. aureus* was introduced. For other test organisms no correction is necessary. The constant in Langmuir's adsorption isotherm was $K=78400\pm8200$ l./mol and $n=3{\cdot}15$ (95% confidence limits: 2·09–5·52).

4. The mean blood concentration was 20·8 μg/ml and the mean pus concentration 17·2 μg/ml in nineteen sets of blood and pus samples. The ratio of pus to blood corresponds to the ratio of published values for the protein concentrations in serum and in inflammatory oedema.

5. It is concluded that for albumin bound drugs the 'storage depot' of the organism also includes the fluid of the tissue spaces including the inflammatory oedema. As recent studies have revealed an extravascular albumin pool similar in size to the plasma pool, this 'storage depot' should not be neglected.

Introduction

Fusidic acid is an example of an antibiotic which is bound to serum protein to a high degree (Godtfredsen, Roholt & Tybring, 1962; Rolinson & Sutherland, 1965). The purpose of the present work was to determine *in vitro* the specificity and the degree of interaction between serum protein and fusidic acid, and to determine *in vivo* the distribution of fusidic acid between blood and pus.

Methods

Electrophoresis

Two samples of 5 μl each were fractionated by zone microelectrophoresis in a 2 mm thick agar gel solidified on microscope slides. A 1% Special Noble Agar (Difco) in 0·05 M barbitone sodium buffer pH 8·4 (Wieme, 1965) was used.

After electrophoresis, the proteins on one slide were precipitated by fixation for 2 min with alcohol: acetic acid: water: 25:5:70 v/v, while the agar on the unfixed slide and a control slide with fusidic acid in barbitone sodium buffer were cut into strips in accordance with the fractions of protein precipitated. The strips were placed in the proper sequence on an N.I.H. agar (Difco) plate inoculated with *Corynebacterium xerosis* (NCTC 9755). The plate was incubated at 35° C for 18 hours.

Quantitative measurement of albumin binding

Any measuring method for the determination of the equilibrium between free and protein bound drug involves a risk of unintentional interference with the equilibrium. Preliminary tests concerning the applicability of the equilibrium dialysis to the determination of the fusidic acid-protein equilibrium confirmed the observation that semipermeable membranes might bind fusidic acid to a very high extent (Barber & Waterworth, 1962), thereby shifting the equilibrium. Based on the generally accepted assumption that only free antibiotic substance is microbiologically active, we therefore chose the decrease in the activity of fusidic acid in media of different albumin content as a measure of the binding.

The fusidic acid concentrations required to obtain 50% growth inhibition (IC50) were determined by serial dilutions in N.I.H. fluid medium containing 0–40 mg/ml of albumin. Fifty per cent growth inhibition is defined as a 50% reduction in turbidity as compared to the control cultures. In our judgment this gives a more precise measure than conventional M.I.C. (Minimum inhibitory concentration). As test organisms, four variants of the same *Staph. aureus* strain (Leo CC178) were used, with sensitivities ranging from 0·04 to 61 μg/ml in albumin-free medium. After inoculation with 10^4 cells/ml the cultures were incubated at 35° C for 18 hours. The validity of this method depends on proper correction being made for any change in sensitivity of the test organisms to free fusidic acid in the presence of albumin.

Fusidic acid assay

Fusidic acid concentrations were determined by an agar-plate diffusion method (N.I.H. agar (Difco) with *C. xerosis* as test organism), diameter of holes, 10 mm or 2·5 mm. Dilution of blood and pus samples with the corresponding standards was made in serum. To make sure that the zones of inhibition measured for serum and pus samples were caused by fusidic acid alone and not influenced by any admixture of other antibiotics or disinfectants, the samples were checked for activity using plates seeded with a fusidic acid resistant variant of *C. xerosis*.

Material

Serum from volunteers and immuno-electrophoretically pure human albumin, produced by preparative electrophoresis and subsequent dialysis (The State Serum Institute, Copenhagen), were used for the tests of protein binding.

The patients were adults, with abscesses, secondary wound infections and fistulous osteomyelitis. During the experimental period the patients were treated with sodium fusidate (Fucidin) 500 mg t.i.d., administered orally. Samples of blood and pus were collected simultaneously, usually after the morning dose. Blood was collected without addition of anticoagulants. The pus was sucked from the bottom of the infection injury into 7 cm long sterilized capillary tubes. Samples of pus were never obtained in connexion with surgical treatment. The pus was never visibly haemorrhagic. The capillary tubes were closed at both ends with wax to ensure minimum evaporation. The samples were kept in ice water until further processing. The capillary tubes were centrifuged on the same day at 900 *g* for 20 minutes. The supernatant was used for assay of fusidic acid and for agar gel microelectrophoresis. A comparison of pus and serum sodium concentration was used as a control of evaporation from the wound surface. The sodium concentration was determined by flame photometry.

Results and Discussion

Electrophoresis

Electrophoresis of serum and pus revealed that albumin was responsible for the serum protein binding of fusidic acid. Some strips of agar between albumin and the electrophoretic localization of free antibiotic were, however, also surrounded by zones of inhibition. This is not an indication of binding to the protein fractions in these strips since electrophoresis performed under the same conditions, but with an antibiotic solution in pure albumin, revealed a pattern identical with the serum electrophoresis. In spite of the brevity of the fractionation procedure (about 30 min), diffusion away from the carrying protein had taken place. Thus, the reversibility of the protein binding makes the electrophoretic method inapplicable to a quantitative determination of the interaction between albumin and fusidic acid.

Quantitative data for albumin binding

If it is assumed that the law of mass action, and consequently Langmuir's adsorption isotherm, is applicable to the interaction, there will be a linear relationship between the albumin concentration (X) and the concentration of total fusidic acid (Y) under conditions where the concentration of free fusidic acid (y) is constant (see Appendix). This dependence has been tested at four different levels of free fusidic acid, using as test organisms four differently sensitive variants of the same

TABLE 1. *Influence of albumin on the* in vitro *activity of fusidic acid against* Staph. aureus

Albumin concentration (mg/ml)	Strain CC178B	CC178H$_1$	CC178A	CC178H$_2$
0	0·0408	0·621	30·6	56·0
0·2	0·0465	0·566	40·1	65·3
0·5	0·0537	0·771	45·0	73·3
1·0	0·0764	1·18	49·6	82·8
2·0	0·140	1·69	62·4	104
5·0	0·234	3·18	89·7	141
10·0	0·498	6·61	157	208
20·0	0·783	7·98	189	225
40·0	1·09	10·5	258	365

Concentration required for 50% inhibition of growth. Mean IC50 in μg/ml.

strain of *Staph. aureus*. At each level, the albumin concentration was varied from 0 to 40 mg/ml and within each albumin concentration the IC50 (total fusidic acid concentration) was determined in serial dilutions. The means corrected for day-to-day variation are shown in Table 1 and the individual determinations in Table 1 of the Appendix.

These values did not lead directly to a linear relationship between the concentration of albumin and the total fusidic acid. As a result of an examination of several modifications of the original model it was necessary to assume that the sensitivity of the test organisms towards fusidic acid was affected by albumin. This influence is, within the series of *Staph. aureus* strains used, correlated with the fusidic acid sensitivity (Table 3 of the Appendix). However, an investigation of the binding with *C. xerosis* (NCTC9755), *Diplococcus pneumoniae* (Leo ED) and *Escherichia coli* (Leo HA2) as test organisms showed no correlation with sensitivity (Table 4 of the Appendix). The explanation may be in the fact that artificially produced resistance to fusidic acid in *Staph. aureus* is followed by a certain attenuation of the micro-organism, as evidenced by, for instance, a decrease in the growth rate. It is thus necessary in the system of fusidic acid-test organism-albumin first to account for the influence of the albumin on the sensitivity of the test organisms to the free antibiotic before describing the fusidic acid-albumin interaction. This influence may be negligible as in the present material when using *E. coli* as the test organism. Subject to this reservation, the method is fully applicable to describing the fusidic acid-albumin interaction and the results are in agreement with the law of mass action.

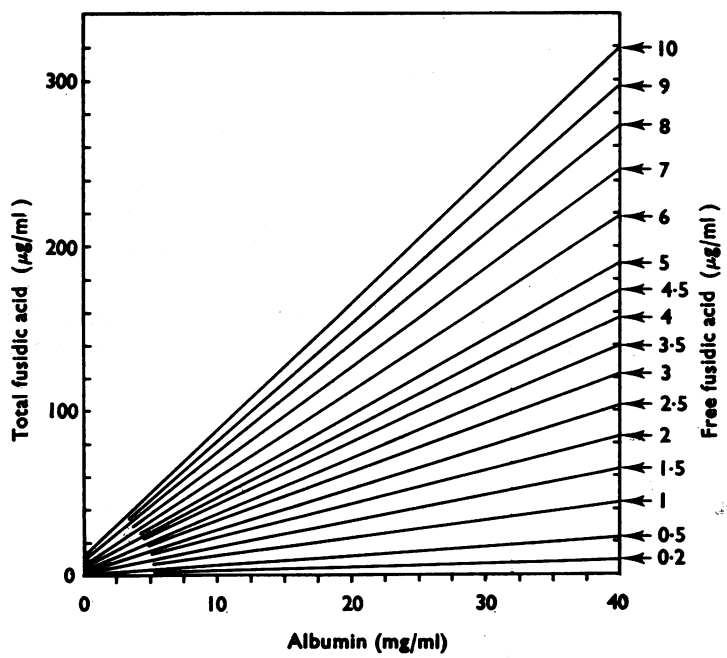

FIG. 1. Relationship between total (Y) and free (y) fusidic acid in μg/ml at varying albumin concentrations (X) in mg/ml.

$$Y=(y)\left(\frac{1+1\cdot136X}{1+0\cdot0462(y)}\right)$$

The binding constants were determined to be: $K=78{,}400\pm8{,}200$ l./mol and $n=3{\cdot}15$, 95% confidence limits=2·09–5·52. Figure 1 has been constructed using these values of the binding constants and shows the relationship between total and free fusidic acid at varying albumin concentration.

Comparative measurements of the protein binding showed that 40 mg/ml of albumin had the same binding effect as pure human serum, whereas no binding could be demonstrated for 10 mg/ml of human gamma globulin. Rolinson & Sutherland (1965) reported 97·2% binding of 50 μg/ml of fusidic acid in human serum. This is in agreement with our results which show 97·7% binding of 50 μg/ml of fusidic acid in the presence of 40 mg/ml of albumin.

If unbound antibiotics can diffuse freely between two compartments of different protein binding capacity, it is generally assumed that at equilibrium the concentrations of free antibiotic are equal in the two compartments. This involves the total concentration being greatest in the compartment containing most antibiotic binding protein (Brodie, 1966). A borderline case is the aqueous humour in the normal intact human eye, which does not contain albumin; the maximum obtainable fusidic acid concentration should therefore be expected to be equal to that of free fusidic acid in the blood stream. Chadwick & Jackson (1969) examined the total fusidic acid concentration in serum and aqueous humour of seventeen patients who had received 500 mg of sodium fusidate t.i.d. for 3 days. The mean concentration in serum was 91·5 μg/ml and in aqueous humour 1·65% of that of serum. Our results showed 3·35% fusidic acid to be present in free form in a medium containing 40 mg/ml of albumin when the total fusidic acid concentration was 91·5 μg/ml. The aqueous humour concentration was thus found to be 70% of the equilibrium concentration. A similar study was performed by Williamson, Russell, Doig & Paterson (1970), who found a relationship between the concentrations in aqueous humour and in serum corresponding to 60% of that of equilibrium after 1 day's treatment and approximately 100% after 2 or 3 days' treatment. The concentrations were determined 12 h after the last dose of sodium fusidate. As expected, particularly high total concentrations of fusidic acid were found in vitreous body and aqueous humour with an increase in protein content associated with prolonged inflammation.

Pus-blood concentrations

The content of fusidic acid was determined in nineteen sets of pus and blood samples from five patients (Table 2). The mean blood concentration was 20·8 μg/ml and the mean pus concentration 17·2 μg/ml, that is 83% of the mean value in blood. Schultze & Heremans (1966) indicate the protein concentration in inflammatory oedema to be as high as 60–80% of the concentration in serum, without essential changes of the individual protein components. If the albumin content of pus is of the same order of magnitude as in inflammatory oedema then the relative amount of fusidic acid in pus of 83% would seem to indicate that the pus concentration of fusidic acid has reached its equilibrium value. That there might be a time lag in the diffusion from blood to pus is indicated by the statistically significant negative correlation between the concentration in blood and the ratio of pus to serum in individual samples, that is the ratio of pus to blood being low at high blood concentrations.

TABLE 2. *Serum and pus concentrations of fusidic acid*

Patient	Sex	Age (years)	Diagnosis	Daily dose of fusidate	Duration of fusidate therapy (days)	Fusidic acid concentrations (μg/ml) Serum	Pus	Ratio of pus to serum
1	F	82	Arteriosclerotic wounds	500 mg×3	2	18·2	12·3	0·68
					4	41·8	23·0	0·55
					7	32·0	22·7	0·71
2	M	57	Traumatic wounds	500 mg×3	1	17·5	29·0	1·66
					4	18·8	23·3	1·24
					7	32·5	28·8	0·89
					11	16·3	20·5	1·26
					14	29·5	16·8	0·57
					19	15·0	27·5	1·83
3	M	37	Ulcera crurum	500 mg×3	2	5·8	4·0	0·69
					4	7·8	14·0	1·79
4	F	63	Osteomyelitis (fistulous)	500 mg×3	2	38·8	20·8	0·54
					3	47·5	19·9	0·42
5	M	44	Osteomyelitis (fistulous)	500 mg×3	9	15·4	8·8	0·57
				500 mg×6	11	4·8	6·8	1·42
					13	14·7	16·5	1·12
					17	18·3	4·6	0·25
					19	3·4	5·3	1·55
					21	15·0	22·3	1·49
					Mean	20·7	17·2	

The alternative explanation of the rather high pus concentration, that evaporation has taken place, is contradicted by the figures for the mean sodium content, 137 mmol/l. and 124 mmol/l. for serum and pus, respectively.

Mouridsen (1970) has demonstrated an accumulation of albumin in wound tissue. If this albumin is entrapped in the crust this may explain the very high concentration of fusidic acid found by Sørensen, Sejersen & Thomsen (1966) in burn crusts.

Our results seem to show that the free fusidic acid is distributed rapidly in the organism and that an equilibrium, determined by the binding capacities of the various compartments, is quickly attained. A contributory cause for the rapid distribution may be the strong lipophilic character of the fusidic acid.

It is generally accepted that albumin bound drugs are not lost to the organism (Brodie, 1966). The albumin binding acts as a drug depot providing a stable, non-toxic concentration of free compound over a prolonged period. Turnover studies of ^{131}I-albumin have revealed an extravascular albumin pool equivalent in size to the total quantity of plasma albumin (Schultze & Heremans, 1966). For albumin bound drugs it would therefore seem reasonable to extend the ' storage depot ' of the organism to include the lymph of the small peripheral vessels and the fluid of the tissue spaces including inflammatory oedema.

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Appendix

Interaction of albumin and fusidic acid

Application of Langmuir's adsorption isotherm to the system: albumin-fusidic acid in aqueous solution, leads to the following relation:

$$(xy)=K\cdot(y)\cdot(n\cdot X-(xy)) \qquad (1)$$

where (xy)=albumin-bound fusidic acid in mol/l.=number of occupied binding sites/litre;
(y)=free fusidic acid in mol/l.;
(X)=total albumin in mol/l.;
n=number of binding sites/mol of albumin
K=equilibrium constant in l./binding site
With $Y=(y)+(xy)$ this may be written:

$$Y=(y)\cdot\left(1+\frac{K\cdot n}{1+(y)\cdot K}\cdot X\right) \qquad (2)$$

Equation (2) predicts a linear relationship between X and Y if (y) is kept constant under varying albumin concentrations. In the present case, we sought to fulfil this condition by determining Y such that the concentration of free fusidic acid, (y), just stopped the growth of a sensitive bacterium, irrespective of the concentration of albumin. Four different variants of the same *Staph. aureus*, with $Y(X=0)$ varying from 0·04 to 61 μg per ml were used in the experiment. The data, expressed as $a=6-\log Y$, are given in Table 1 for different X and different dates.

TABLE 1. *Influence of albumin on the* in vitro *activity of fusidic acid against* Staph. aureus

Strain	Albumin conc. (mg/ml)	Dates								
		24/6	18/7	24/7	31/7	30/8	9/9	11/12	17/12	8/1
CC178B	40	5·75				5·8	5·8	6·1	6·3	6·2
	20	6·0					5·9	6·2	6·4	6·3
	10	6·25	6·25	6·2	6·2			6·3	6·6	6·5
	5	6·6	6·5	6·6	6·6			6·7	6·9	6·7
	2	6·8	6·9	6·75	6·8			6·9	7·2	6·8
	1	7·1	7·1	7·1	7·1			7·2	7·3	7·1
	0·5	7·3	7·2	7·2	7·2					
	0·2	7·4	7·3	7·25	7·2					
	0	7·5	7·3	7·4	7·3	7·3	7·4	7·4	7·6	7·3
									7·6	7·5
CC178H$_1$	40					4·8	4·9	5·1	5·3	5·0
	20						5·1	5·2	5·3	5·1
	10		5·2	5·0	5·2			5·3	5·4	5·2
	5		5·5	5·3	5·5			5·6	5·8	5·5
	2		5·7	5·7	5·7			5·75	6·2	5·8
	1		6·0	5·8	5·9			6·0	6·2	5·9
	0·5		6·1	6·0	6·1					
	0·2		6·2	6·2	6·2					
	0		6·2	6·2	6·2	6·2	6·2	6·25	6·25	6·2
									6·4	6·3
CC178A	40					3·5	3·6	3·6	3·7	3·75
	20						3·8	3·8	3·8	3·8
	10		3·8	3·75	3·8			3·9	3·9	3·9
	5		4·1	3·8	4·0			4·2	4·2	4·2
	2		4·25	4·1	4·2			4·3	4·3	4·3
	1		4·3	4·2	4·25			4·3	4·5	4·5
	0·5		4·4	4·25	4·25					
	0·2		4·5	4·25	4·3					
	0		4·6	4·3	4·4	4·4	4·35	4·65	4·7	4·7
									4·7	4·7
CC178H$_2$	40					3·4	3·5	3·4	3·5	3·6
	20						3·7	3·7	3·7	3·8
	10			3·6	3·6			3·8	3·8	3·8
	5			3·75	3·7			3·9	4·0	4·1
	2			3·8	3·8			4·1	4·2	4·2
	1			3·9	4·0			4·2	4·2	4·3
	0·5			4·0	4·1					
	0·2			4·1	4·1					
	0			4·1	4·1	4·3	4·2	4·3	4·4	4·4
									4·4	4·4

Individual IC50 expressed as a =6−log (μg/ml.)

Inspection of Table 1 reveals that the level of a-values varies significantly from day to day in a way independent of X. Correction for this leads to mean values given in Table 2 together with the number of data contributing to this mean.

The best estimate of the variance within the same strain and albumin dose is: $s^2(a)=0{\cdot}00729$ (after correction for day-to-day variation).

The values of Table 2 do not lead to straight lines when Y is plotted against X. For large X the experimental Y values are much smaller than expected. Various mathematical models have been developed in order to explain this discrepancy, including some involving association between albumin molecules or binding sites of different strength, but, so far, the only one leading to a satisfactory agreement with the experimental values is the following:

(y) is taken to be, not a constant equal to $Y(X=0)$ but to $Y(X=0)\cdot\exp(-k_s\cdot X)$ and the relation between X and Y becomes:

$$Y=Y_0\cdot(\exp(-k_s\cdot X)+K\cdot n\cdot X/(\exp(k_s\cdot X)+K\cdot Y_0)) \quad (3)$$

Taking logarithms on both sides of (3) and rearranging:

$$a=\mu+bX+\log(1+c\cdot 10^{6-\mu-bX})-\log(1+c\cdot 10^{6-\mu-bX}+d\cdot X) \quad (4)$$

$\mu=6-\log Y_0$, $b=0{\cdot}4343k_s/6{\cdot}9\cdot 10^4$ (albumin in mg/ml), $c=K/5{\cdot}4\cdot 10^5$ (fusidic acid in μg/ml), $d=K\cdot n/6{\cdot}9\cdot 10^4$.

a may be assumed to be normally distributed about the expected value and c and d should be the same for all four series. Maximum likelihood determination of the parameters leads to the values shown in Table 3.

Common to all series:

$K=24{,}900\pm 5{,}200$ (1 per binding site) or $K=78{,}400\pm 8{,}200$ l./mol

$n=3{\cdot}15$, 95% confidence limits: 2·09–5·52.

Insertion of these values in (4) and calculation of the deviations between the a-values calculated from (4) and the experimental values leads to an overall estimate of the variance of a: $s^2(a)=0{\cdot}0129$ $f=26$ ($s(a)=0{\cdot}11$). This is significantly higher than the estimate $s^2(a)=0{\cdot}00729$ $f=154$ obtained for a, within the same strain, albumin concentration and date, but less than the value $s^2(a)=0{\cdot}0185$ within the same strain

TABLE 2. *Influence of albumin on the* in vitro *activity of fusidic acid against* Staph. aureus

Albumin concentration (mg/ml)	CC178B N	CC178B a	CC178H$_1$ N	CC178H$_1$ a	CC178A N	CC178A a	CC178H$_2$ N	CC178H$_2$ a
0·0	11	7·389	10	6·207	10	4·514	9	4·252
0·2	4	7·333	3	6·247	3	4·397	2	4·185
0·5	4	7·270	3	6·113	3	4·347	2	4·135
1·0	7	7·117	6	5·930	6	4·305	5	4·082
2·0	7	6·853	6	5·772	6	4·205	5	3·982
5·0	7	6·631	6	5·497	6	4·047	5	3·852
10·0	7	6·303	6	5·180	6	3·805	5	3·682
20·0	5	6·106	4	5·098	4	3·723	4	3·648
40·0	6	5·963	5	4·978	5	3·588	5	3·438

Mean values of IC50 corrected for day-to-day variation, expressed as $a=6-\log$ (μg/ml).

TABLE 3. *Maximum likelihood determinations of IC50 in albumin-free substrate* (Y) *and* k_s *for* Staph. aureus

Strain	$Y(X=0)$ (μg/ml)	k_s(mol^{-1})	k_s(ml/mg)
CC178B	0·0396±0·0023	837±225	0·0121±0·0033
CC178H$_1$	0·569±0·034	1573±245	0·0228±0·0036
CC178A	33·2±2·0	2697±310	0·0391±0·0045
CC178H$_2$	61·0±4·0	2967±350	0·0430±0·0051

and albumin concentration, without correction for date. It follows that (4) is an adequate description of the data.

The model from which (4) is derived implies that the observed deviation from Langmuir's adsorption isotherm is due to the bacterium and not to the system albumin-fusidic acid. It places no *a priori* restriction whatsoever on the relation between $Y(X=o)$ and k_S. For the above four strains an apparent relation exists: k_S increases with increasing resistance against fusidic acid. A possible explanation may be found in the observation that in the present series of variants of the same *Staph. aureus*, increase in resistance against fusidic acid is followed by a decrease in growth rate. In order to check that k_S is not generally an increasing function of $Y(X=o)$ a search was instigated for bacteria with different 'natural' resistance, growth rates and k_S values. The results are given in Table 4.

TABLE 4. *Maximum likelihood determinations of IC50 in albumin-free substrate (Y) and k_S for different test organisms*

	Strain	$Y(X=o)$ (μg/ml)	k_S (mol^{-1})	k_S (ml/mg)
Corynebacterium xerosis	NCTC 9755	0·0114±0·0014	2,194±317	0·0318±0·0046
Diplococcus pneumoniae	Leo EA	12·4±2·0	4,718±549	0·0684±0·0080
Escherichia coli	Leo HA2	613±69	−694±513	−0·0101±0·0074

The figures were calculated using the above values for K and n.

It follows that k_S is characteristic for the bacteria used in the experiment. If *E. coli* had been chosen for the initial investigation, no deviation from Langmuir's adsorption isotherm would have been observed.