Some observations on the penetration of antibiotics through mucus *in vitro*

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SYNOPSIS   A study has been made on the penetration values *in vitro* of antibiotics through hog gastric mucin at *p*H 7·4. Determinations have also been done on the binding of antibiotics to mucus and human plasma. The mucolytic agent N-acetyl cysteine has been studied in regard to its effect on antibiotics penetrating mucus and for any possible inactivation of antibiotics.

The chemotherapeutic treatment for cystic fibrosis patients has for some years utilized a wide range of antibiotics in the control and prevention of bacterial chest infections. A knowledge of the rate at which antibiotics diffuse into mucus and the concentrations of antibiotics obtained therein would be helpful in the management of cystic fibrosis. There are practical difficulties in assessing the clinical effect of one of a number of variables, which may include the use of one or more antibiotics, physiotherapy, and mucolytic agents, against the variable natural history of each inflammatory episode. The investigations reported here were designed to determine the effectiveness of antibiotics in penetrating mucus *in vitro*, by simulating conditions which would be expected when giving the antibiotics by aerosol or via the blood stream in the hope of giving a more accurate assessment of relative antibiotic penetration values. Experiments were also done to determine the effect of the mucolytic agent N-acetyl cysteine (Airbron) on antibiotic penetration values.

MATERIALS AND METHODS

TEST ANTIBIOTICS   A wide range of antibiotics commonly used to combat both Gram-positive and Gram-negative bacteria was studied. These were ampicillin, cephalosporin (Ceporin), cloxacillin, erythromycin gluceptate, fucidin, lincomycin, methicillin, neomycin, novobiocin, penicillin G, polymyxin B, quinacillin, streptomycin, sulphadiazine, sulphamezathine, and tetracycline hydrochloride.

TEST MUCIN   Hog gastric mucin (L. Light & Co.) was used for all the general tests. The mucin powder was made up in 0.1M *p*H 7·4 phosphate buffer (Sorensen) at concentrations from 1 to 20% (w/v), well mixed and incubated for one hour at 37°C. before use.

MUCOLYTIC AGENT   N-acetyl cysteine (Airbron, B.D.H. Ltd.) at 5% (w/v) was the only mucolytic agent used in this series of experiments.

DETERMINATIONS ON THE ANTIBIOTIC-TO-MUCUS BINDING These experiments were done before those to measure the penetration of antibiotics into mucus, in order that any antibiotic binding could be considered in the evaluation of the penetration values. The technique used was basically that of Bond, Lightbown, Barber, and Waterworth (1963) which separates free antibiotic from protein-bound antibiotic by means of ultrafiltration through a Visking membrane under negative pressure. A 20 ml. solution of the test antibiotic was made up at 10 μg./ml. in 0.1M *p*H 7·4 phosphate buffer and divided into two equal portions A and B. Hog gastric mucin was added to volume A to give a concentration of 1% (w/v). The antibiotic/mucus mixture and the antibiotic solution volume B were incubated at 37°C. for one hour before use. Volume A was then ultrafiltered for six hours at a negative pressure of 120 mm. The ultrafiltrate produced was divided into two portions: one portion was checked for the presence of protein by a biuret test, and if negative the second portion was assayed for antibiotic content by a cup plate assay against standards made up from the antibiotic solution volume B. If the ultrafiltrate contained any protein it was discarded and the experiment was repeated. The organisms used in the microbiological assays of the antibiotics were those recommended by the National Collection of Type Cultures (N.C.T.C.). The test sulphonamides could not be assayed in this way due to assay difficulties, so the sulphonamide ultrafiltration tests were run at 1,000 μg./ml. and the ultrafiltrate was assayed by the chemical method of Bratton and Marshall (1939). The percentage binding of the antibiotics to hog mucin was determined from the differences between the antibiotic content of the ultrafiltrate and the original 10 μg./ml. solution. When the hog mucin showed evidence of antibiotic binding, ultrafiltration experiments were also done using fibrocystic mucus at 1% (w/v).

PROTEIN BINDING TO HUMAN PLASMA   The protein binding of the antibiotics to pooled human plasma from

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20 normal adults was determined by the ultrafiltration technique. The test antibiotics were made up at 10 μg./ml. in pH 7·4 phosphate buffer for standard controls, and in the pooled human plasma for ultrafiltration. The suggestion of Bond (1964) that at least 95% serum should always be present in protein-binding determinations was observed in all experiments. The pooled human plasma tested was then used as a vehicle for supplying antibiotics in the plasma to mucus diffusion experiments.

N-ACETYL CYSTEINE INACTIVATION OF ANTIBIOTICS N-acetyl cysteine has been found to inactivate some antibiotics (Lawson and Saggers, 1965), and as experiments using N-acetyl-cysteine-treated mucus would be done in this survey, further tests were carried out to determine if any of the test antibiotics were inactivated. The antibiotics were tested by titrating them against standard assay organisms (N.C.T.C.) with and without 5% (w/v) N-acetyl cysteine in the medium. Differences in the minimal inhibitory concentrations of the antibiotics to the test organisms due to the presence of N-acetyl cysteine were noted. The above technique was not suitable for testing inactivation of sulphonamides as the 5% N-acetyl cysteine reacted with the laked blood added to the medium as a desensitizing agent. The effect of N-acetyl cysteine on the sulphonamide drugs was therefore determined using the chemical assay method of Bratton and Marshall (1939). Sulphonamide solutions were used at 1,000 μg./ml. in pH 7·4 phosphate buffer with and without 5% N-acetyl cysteine and then tested for free and conjugated sulphonamide drug.

MEASUREMENT OF ANTIBIOTIC DIFFUSION THROUGH MUCIN A microdiffusion apparatus designed specifically for the purpose of measuring the diffusion of antibiotics through 1 mm. columns of mucin (Saggers, 1966) was used in all diffusion experiments. The effect of hog mucin solutions at 1, 5, 10, 15, and 20% (w/v) on antibiotic penetration was determined by measuring the amount of antibiotic that diffused through a 1 mm. column of hog mucin in 24 hours at 37°C. by microbiological assay. The effect of N-acetyl cysteine at 5% (w/v) on the diffusion rates of antibiotics through hog mucin was also studied. The hog mucin for these experiments was prepared by incubating the mucin in a 5% solution of N-acetyl cysteine in pH 7·4 phosphate buffer for one hour at 37°C.

Antibiotic concentrations of 200 μg./ml. in pH 7·4 phosphate buffer were used to study the effect of aerosol to mucus penetration and at 8 μg./ml. in plasma to study blood to mucus penetration.

RESULTS

MUCUS TO ANTIBIOTIC BINDING   Neomycin, novobiocin, polymyxin B, streptomycin, and the tetracyclines were the only antibiotics to show binding to 1% hog mucin (Table I). The tetracyclines all formed an orange-yellow pigmented complex with the hog mucin, which was insoluble in pH 7·4 phosphate buffer. Albert (1952, 1956) found that the tetracyclines formed orange-yellow chelation compounds

TABLE I
BINDING OF ANTIBIOTICS TO 1% (w/v) HOG MUCIN AT pH 7·4

Antibiotic	Assay Organism	Percentage of Mucus-bound Antibiotic
Ampicillin	Sarcina lutea	0
Cephalosporin	Sarcina lutea	0
Chlortetracycline	Bacillus pumilus	100
Cloxacillin	Sarcina lutea	0
Erythromycin	Sarcina lutea	0
Fucidin	Oxford staphylococcus	0
Lincomycin	Sarcina lutea	0
Methicillin	Sarcina lutea	0
Oxytetracycline	Klebsiella pneumoniae	72·5
Neomycin	Bacillus pumilus	94·0
Novobiocin	Bacillus subtilis	30
Paromomycin	Bacillus subtilis	0
Penicillin G	Sarcina lutea	0
Polymyxin B	Bordatella bronchiseptica	25·0
Streptomycin	Bacillus subtilis	25·0
Sulphadiazine	Colorimetric	Conjugated
Sulphamezathine	Colorimetric	Conjugated
Tetracycline HCl	Bacillus pumilus	87·5

in the presence of certain metal ions. The possibility of chelation occurring with hog mucin was investigated by pre-chelating the metal ions in the mucin with 0·3M ethylene diamine tetracetic acid (E.D.T.A.) before the addition of the tetracyclines. The results of the mucus to tetracyclines binding experiments in the presence of E.D.T.A. (Table II) show a reduction in the binding of hog mucin.

TABLE II
EFFECT OF 0·3M E.D.T.A. ON BINDING OF TETRACYCLINES TO HOG MUCIN

Antibiotic	Percentage Binding to Hog Mucin	
	Without E.D.T.A.	With 0·3 M E.D.T.A.
Chlortetracycline	100	66
Oxytetracycline	72·5	45
Tetracycline HCl	87·5	20

Ultrafiltration experiments were repeated using 1% (w/v) fibrocystic mucus with the antibiotics that showed binding to hog mucin. These experiments showed that antibiotic to mucus binding also occurred with fibrocystic mucus (Table III), but in different proportions.

TABLE III
BINDING OF ANTIBIOTICS TO 1% (w/v) FIBROCYSTIC MUCUS AT pH 7·4

Antibiotic	Assay Organism	Percentage of Mucus-bound Antibiotic
Neomycin	Bacillus pumilus	50
Novobiocin	Bacillus subtilis	40
Polymyxin B	Bordatella bronchiseptica	Not tried
Streptomycin	Bacillus subtilis	100
Tetracycline HCl	Bacillus pumilus	5

PLASMA BINDING OF THE ANTIBIOTICS   The results of the experiments to determine the protein binding of the test antibiotics to pooled human plasma were in the range of those expected for the test antibiotics.

EFFECT OF N-ACETYL CYSTEINE ON ANTIBIOTICS   Lawson and Saggers (1965) found that ceporin, the penicillins, and tetracycline hydrochloride were in-activated by 5% (w/v) N-acetyl cysteine. The effect of N-acetyl cysteine on these antibiotics was not re-investigated in this series of experiments. Neomycin and paromomycin were the only test antibiotics inactivated by 5% (w/v) N-acetyl cysteine (Table IV).

TABLE IV

EFFECT OF N-ACETYL CYSTEINE ON ANTIBIOTICS

Test Antibiotic	Minimal Inhibitory Concentrations (μg./ml.)	
	Without N-acetyl Cysteine	With N-acetyl Cysteine
Lincomycin	0·1	0·1
Neomycin	1–2	2–5
Novobiocin	1–2	1–2
Paromomycin	1–2	10
Polymyxin B[1]	40	10
Streptomycin	0·1–0·5	0·1–0·5
Sulphadiazine[2]	—	100% conjugated
Sulphamezathine[2]	—	100% conjugated

[1]Assayed using *Pseudomonas pyocyanea*
[2]Assayed by chemical assay

Polymyxin B, using *Pseudomonas pyocyanea* as the assay organism, showed a reduction in the minimal inhibitory concentration of 30 μg./ml. in the presence of 5% (w/v) N-acetyl cysteine. True synergism between polymyxin B and N-acetyl cysteine could not be demonstrated using conven-tional microbiological techniques, so investigations were done to determine if it altered the physical characteristics of the bacterial growth medium. The *p*H of the medium was unaffected by the addition of 5% (w/v) N-acetyl cysteine, but the E h potential was reduced to +0·227 mv., which was more reduced than a 24-hour broth culture of *Ps. pyocyanea*. The test medium plus 10% (w/v) N-acetyl cysteine was found to inhibit the growth of *Ps. pyocyanea* without polymyxin B being present, so the apparent synerg-ism of the polymyxin B and N-acetyl cysteine was due to a combination of the inhibitory effects of the antibiotic and mucolytic agent.

The chemical assays for the inactivation of the sulphonamides with N-acetyl cysteine showed that complete conjugation of the sulphonamide drugs occurred after one hour at 37°C.

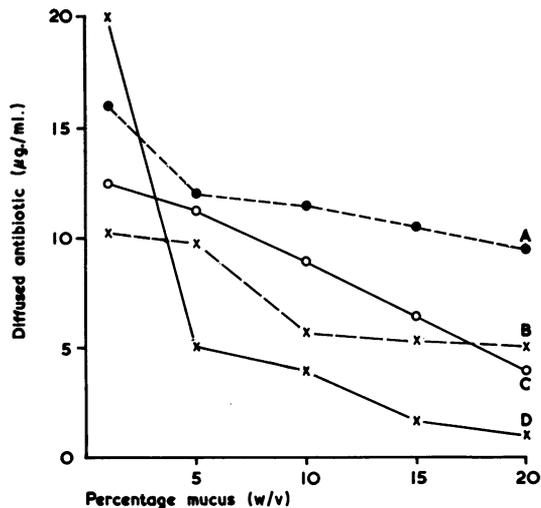AEROSOL PENETRATION OF ANTIBIOTICS THROUGH HOG MUCIN   The concentrations of the antibiotics that

FIG. 1.   *The concentrations of antibiotics obtained after diffusion through 1 mm. columns of 0 to 20% hog mucin.*
A   *Lincomycin*
B   *Tetracycline HCl*
C   *Fucidin*
D   *Penicillin G*

diffused through 1 mm. columns of hog mucin did not decrease linearly with an increase in column mucus concentration. The concentration of anti-biotic obtained after diffusion through 1% hog mucin was in most cases much higher than that obtained after diffusion through 5% hog mucin (Fig. 1). The initial drop in the antibiotic concentra-tion between 1% and 5% hog mucin was not main-tained; mucus concentrations above 5% produced a gradual decrease in antibiotic concentration as the concentration rose in the column of mucus. Tetra-cycline and fucidin were both exceptions to this; tetracycline showed only a small initial drop in con-centration, due to the antibiotic chelating with the metal ions in the mucin, and the penetration of the mucus by fucidin was nearly linear with mucus concentration.

The concentrations of the antibiotics obtained after diffusion through 1 mm. columns of 15% (w/v) hog mucin (Table V) were compared with their molecular weights and chemical structures in order to determine some relationship between antibiotic penetration and structure. No direct correlation between chemical structure and the concentrations of the antibiotics obtained after diffusion could be demonstrated. The molecular weights of the anti-biotics, however, were correlated to some extent with the concentrations obtained after diffusion: the higher the molecular weight, the more the antibiotic diffused through the mucus column.

TABLE V

CONCENTRATIONS OF ANTIBIOTICS OBTAINED AFTER DIFFUSION THROUGH 1 MM. COLUMNS OF pH 7·4 15% HOG MUCIN FOR 24 HOURS AT 37°C.

Antibiotic	Molecular Weight (approx.)	Concentration of Antibiotic after Diffusion (μg./ml.)
Sulphadiazine[1]	272	120·0
Sulphamezathine[1]	278	52·0
Penicillin G	356	3·4
Ampicillin	372	1·7
Cephalosporin	415·5	3·2
Methicillin	426	3·6
Quinacillin	435	2·5
Cloxacillin	442·5	4·5
Tetracycline HCl[2]	444·43	5·4
Lincomycin	455	10·5
Fucidin	539	7·2
Streptomycin[2]	581·58	5·0
Neomycin[2]	598	None detected
Novobiocin[2]	612·65	7·0
Paromomycin	645	None detected
Erythromycin	960	16·0
Polymyxin B[2]	1176	None detected

[1]Run at 1,000 μg./ml.
[2]Bound to mucin

The antibiotic diffusion experiments were also run using 15% (w/v) hog mucin pretreated with 5% (w/v) N-acetyl cysteine. The results (Table VI) show that antibiotic diffusion was considerably reduced in all cases. When the antibiotic was inactivated by N-acetyl cysteine, e.g., penicillin G, no antibiotic was detected after diffusion.

TABLE VI

CONCENTRATIONS OF ANTIBIOTICS OBTAINED AFTER DIFFUSION FOR 24 HOURS AT 37°C. THROUGH 1 MM. COLUMNS OF pH 7·4 15% HOG MUCIN PRETREATED WITH 5% (W/V) N-ACETYL CYSTEINE

Antibiotic	Molecular Weight (approx.)	Concentration of Antibiotic after Diffusion (μg./ml.)
Penicillin G[1]	356	None detected
Ampicillin[1]	372	None detected
Cephalosporin[1]	415·5	None detected
Methicillin[1]	426	None detected
Quinacillin[1]	435	None detected
Cloxacillin[1]	442·5	None detected
Tetracycline HCl[2]	444·43	Trace detected
Lincomycin	455	1·2
Fucidin	539	0·9
Streptomycin[2]	581·58	None detected
Neomycin[1,2]	598	None detected
Novobiocin[2]	612·65	0·4
Paromomycin[1]	645	None detected
Erythromycin	960	5·0
Polymyxin B[2]	1176	None detected

[1]Inactivated by 5% (w/v) N-acetyl cysteine
[2]Bound to mucin

PLASMA TO MUCUS PENETRATION THROUGH HOG MUCIN None of the antibiotics tested diffused through hog mucin at concentrations above 1% (w/v) mucin.

DISCUSSION

MUCUS BINDING OF ANTIBIOTICS The binding of antibiotics to mucus is important as it will determine the proportion of free antibiotic available for diffusion. The bound antibiotic, apart from being bacteriologically inactive, would not readily diffuse into the surrounding environment (Rolinson, 1964) which would be an important consideration in the treatment of cystic fibrosis. The tetracyclines were partially bound to the mucus by chelation with the metal ions it contained. Chernick and Barbero (1959) found that tracheo-bronchial secretions in cystic fibrosis had a comparatively high calcium content and this would affect the activity of the tetracycline group of antibiotics. The differences between the binding of the test antibiotics to hog and fibrocystic mucins can be accounted for by the variations in the chemical structures of the test mucins.

DIFFUSION OF ANTIBIOTICS THROUGH 1 MM. COLUMNS OF HOG MUCIN A number of points are discussed here concerning the structure of the antibiotics and their possible relationships to their penetrability.

The penicillin group of antibiotics after diffusion through 15% hog mucin gave penetration values of 3·2 to 4·5 μg./ml. with the exceptions of ampicillin and quinacillin, which do not differ radically from the other penicillins in their chemical structures. The only major difference shown by these two antibiotics is that they carry different electrical charges at normal pH than the other test penicillins. The majority of penicillin molecules are polarized at normal pH, having one negative charge on the carboxyl group attached to the thiazolidine ring system. Ampicillin, however, also has a charged amino group on the side chain making the molecule electrically neutral; quinacillin has a negatively charged carboxyl group on the side chain giving the molecule a double negative charge. The different electrical charges on these molecules may account for their relatively low penetration values through hog mucin.

The antibiotic lincomycin, which is chemically distinct from all the other test antibiotics, gave the second highest mucus penetration value. No structural reason for this was evident.

Erythromycin, fucidin, and novobiocin all contained benzene ring systems linked by common carbon atoms. These antibiotics all gave good penetration values. The amounts of these antibiotics that penetrated the mucus column were all above 7 μg./ml. Whether the structural similarity is significant is debatable.

Neomycin, paromomycin, and streptomycin are all derived from various sugars. Streptomycin was

the only one of this group that penetrated the mucus but there was no apparent reason for this.

Neomycin, novobiocin, polymyxin B, streptomycin, and tetracycline were all bound to hog mucin, which would have reduced their penetration values. Penetration of the 15% hog mucin could not be detected in the cases of neomycin and polymyxin, but for the other bound antibiotics good penetration values were obtained.

The larger antibiotic molecules diffused more rapidly through 15% hog mucin than the small antibiotic molecules. It seems possible that some form of gel filtration was taking place in the mucus, the smaller antibiotic molecules becoming trapped in pores in the cross-linked mucopolysaccharides and the larger molecules being unable to pass into the pores in the mucin and so diffusing round the outside of the mucus plug.

The action of N-acetyl cysteine on the penetration of antibiotics through 15% hog mucin caused a reduction in the penetration values obtained. A possible reason for the lower antibiotic penetration values is that the N-acetyl cysteine breaks down the cross linkages in the mucus causing slower diffusion.

CLINICAL APPLICATIONS OF THESE FINDINGS The penetration of mucus by aerosolized antibiotics may not be important in the treatment of lung infection itself, as these areas can be effectively reached via the blood stream. It is, however, desirable to sterilize mucus in the respiratory tract and to this end a combination of antibiotic therapy via the blood stream and by aerosol may be used. When choosing an antibiotic for use in an aerosol, next to its antibacterial properties consideration should be given to its mucus penetrability, bearing in mind that high molecular weight antibiotics have the best penetration rates. Antibiotics which are bound to mucus will not be very effective as antibacterial agents and the use of these should be avoided if possible. These conditions when fulfilled should lead to a more effective control of infected sputum.

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