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The diffusion of penicillin G and ampicillin through phospholipid sols

JOHN M. PADFIELD* AND I. W. KELLAWAY

Pharmaceutics Research Unit, University of Nottingham, U.K.

The self-diffusion coefficients of penicillin G and ampicillin have been determined at 25° in water and in the presence of a swamping concentration of electrolyte. The antibiotics diffused through phospholipid dispersions at a reduced rate due to interaction with the lipid aggregates. Ampicillin diffused through the phosphatidylcholine and phosphatidylethanolamine dispersions more rapidly than penicillin G, whereas the latter diffused more rapidly through the lysophosphatidylcholine dispersions. Estimates of binding have been made from these data and compared with those obtained from equilibrium dialysis studies. Surface tension measurements indicated that the antibiotics exhibited minimal surface activity. These results have been correlated with data obtained in other studies and a possible explanation has been advanced for the reported differences in *in vivo* activity of penicillin G and ampicillin.

A modification of the open-ended capillary tube technique for the determination of self-diffusion coefficients of low-energy β-emitters (e.g. [^{14}C]) has been described (Kellaway & Padfield, 1973). This report describes the application of the technique to a study of the diffusion of penicillin G and ampicillin through phospholipid sols. These antibiotics are known to exhibit marked differences in *in vivo* activity (Stewart, Coles & others, 1961) and this work forms part of an integrated study designed to investigate this phenomenon (Padfield, 1972).

Castleden & Fleming (1966, 1970) have examined the diffusion of ions through protein and phospholipid sols by means of a continuous monitoring apparatus, and succeeded in forming a lipoprotein interface in the capillary tube. Saunders (1963) found that sodium and potassium chlorides moved more slowly through a lipoprotein interface than in aqueous solution, and that addition of calcium chloride reduced the rate further.

Dialysis has been widely used to investigate binding to macromolecules. Patel & Foss (1964, 1965) have described an equilibrium dialysis method for assessing the binding of preservatives to polyoxyethylene sorbitans and the technique has been extended to protein binding using Plexiglass cells with cellulose membranes (Patel, Sheen & Taylor, 1968; Cho, Mitchell & Pernarowski, 1971). We reported a dynamic dialysis study of penicillin-phospholipid interaction (Padfield & Kellaway, 1973a).

MATERIALS AND METHODS

Materials

Potassium penicillin G was the gift of Glaxo Research Limited, Greenford. [^{14}C] penicillin G was obtained from The Radiochemical Centre, Amersham. Sodium ampicillin and [^{14}C]ampicillin were the gifts of Beecham Research Laboratories,

* Present address: School of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, U.K.

Betchworth. The materials were laboratory reference materials and were used as supplied. Analar sodium chloride was used as supplied. All other materials were used as previously described (Kellaway & Padfield, 1973).

Preparation of phospholipids and phospholipid dispersions

This was as previously described (Padfield & Kellaway, 1973a).

Surface tension

The effect of critical micelle concentration (cmc) on the determination of self-diffusion coefficients has been previously discussed (Kellaway & Padfield, 1973). Penicillin G has been ascribed a cmc by some authors (e.g. Hauser, Phillips & others, 1949; McBain, Huff & Brady, 1949; Thakkar & Wilham, 1970), whereas others (e.g. Lund & Pedersen-Bjergaard, 1949; Goyan, 1949; Hocking, 1951; Few & Schulman, 1953), have not observed surface activity. Surface tension measurements were, therefore, made on solutions of penicillin G and ampicillin in triple-distilled water; 0·05, 0·10, 0·25 and 0·50% w/v solutions of the antibiotics were examined for surface activity using a Du Nouy Tensiometer (Cambridge Instruments Limited), but in no case was significant surface activity detected.

Diffusion

The modified open-ended capillary technique and the general method previously described (Kellaway & Padfield, 1973) were used; the apparatus was calibrated with ^{22}Na Cl and [^{14}C]DL-α-alanine. A PPO-dioxane-xylene liquid scintillator (Padfield, 1972) was added to the samples before determining the radioactivity on a Packard TriCarb liquid scintillation spectrometer, model 3380.

The presence of a fast-moving counterion to the benzylpenicillanate ion, i.e. K^+, or to the α-aminobenzylpenicillanate ion, i.e. Na^+, would be expected to result in a decreased self-diffusion coefficient of the large anion (Stigter, Williams & Mysels, 1955). Experiments were made to investigate the effect of a swamping concentration of electrolyte in the bath solution.

Lysophosphatidylcholine was used to determine the effect of phospholipid in the bath solution at a concentration equal to that in the capillary. As no increase in the diffusion coefficient of either antibiotic was detected in the absence of this phospholipid from the bath, phospholipid was omitted from all the inactive bath solutions.

Concentrations of 0·5mM antibiotic and 0·05mM phospholipid were used with a swamping concentration (0·1M) of electrolyte. The mixtures were allowed to stand for 6 h after preparation, the capillaries filled and placed in the inactive solution thermostated at 25° ± 0·01°.

The equations for calculating the diffusion coefficients have been described by Kellaway & Padfield (1973) and fully discussed by Castleden & Fleming (1966).

Equilibrium dialysis

The apparatus used was similar to that described by Humphreys & Rhodes (1968). The Perspex donor and recipient cells (volume 30 ml) were separated by a cellulose acetate membrane (Visking) and stirring was effected by bar magnets activated by immersible control units. The temperature was controlled at 25 ± 0·01°. The donor cell contained the 0·5 mM antibiotic-0·05 mM phospholipid mixture, while the recipient cell contained triple-distilled water. Sampling was performed at intervals of 8 h until equilibrium was attained. This was usually 64 h.

RESULTS AND DISCUSSION

The values in Table 1 for the self-diffusion of the antibiotics are similar to those reported by Gel 'Perin, Klyueva & Ainshtein (1960) for kanamycin ($3{\cdot}95 \times 10^{-10}$ m^2 s^{-1}) (mol. wt 484) and monomycin ($3{\cdot}40 \times 10^{-10}$m^{2}s^{-1}). The quoted values are mean values of the diffusion coefficient D each calculated from the slope of the line of five separate determinations, individually subjected to linear regression analysis. As the only structural difference between penicillin G and ampicillin is the presence of an α-amino group on the side chain of the latter, the more rapid diffusion of ampicillin may reflect changes in the rotation of the side chain, with possible "streamlining" of the molecule due to restricted rotation.

Table 1. *Diffusion coefficients of antibiotics in the presence of phospholipids*[a].

System	D(x 10^{-10}m^{2}s^{-1}) ± standard error[b]	Correlation coefficient range	$\frac{s^2 \max^c}{s^2 \min}$
Pen G	$4{\cdot}01_2 \pm 0{\cdot}04$	0·916–0·986	3·868
Pen G/KCl	$3{\cdot}81_5 \pm 0{\cdot}04$	0·931–0·978	2·432
Pen G/KCl/PC	$3{\cdot}41_3 \pm 0{\cdot}06$	0·927–0·986	2·817
Pen G/KCl/LPC	$3{\cdot}63_8 \pm 0{\cdot}04$	0·931–0·964	2·111
Pen G/KCl/PE	$3{\cdot}51_8 \pm 0{\cdot}08$	0·925–0·991	2·061
Amp	$4{\cdot}58_1 \pm 0{\cdot}05$	0·919–0·965	2·526
Amp/NaCl	$4{\cdot}54_2 \pm 0{\cdot}02$	0·928–0·962	3·791
Amp/NaCl/PC	$4{\cdot}25_3 \pm 0{\cdot}04$	0·942–0·973	1·998
Amp/NaCl/LPC	$3{\cdot}29_8 \pm 0{\cdot}06$	0·931–0·967	1·987
Amp/NaCl/PE	$4{\cdot}48_9 \pm 0{\cdot}06$	0·942–0·971	2·003

Pen G = Potassium Penicillin G (0·5 mM). Amp = Sodium Ampicillin (0·5 mM). PC = Phosphatidylcholine. LPC = Lysophosphatidylcholine. PE = Phosphatidylethanolamine. (all 0·05 mM).

a. variance ratio and Student's *t* tests were performed on all data, and significant differences were found between antibiotic-phospholipid systems and, more significantly, between penicillin G and ampicillin systems. b. Confidence interval = 95%. c. Ratio of maximum: minimum residual mean squares within the data sets.

Since penicillins are known to be unstable to aqueous solution (Hou & Poole, 1971), the possibility of degradation in this extended work could not be ignored, so an ultraviolet absorption method was used throughout the work to ensure that the undegraded species were being measured (Padfield, 1972).

The breakdown products of penicillin G were observed to have an increased diffusion coefficient ($6{\cdot}02_9 \times 10^{-10}$ m^{2}s^{-1}). The primary product of acid degradation is penillic acid (Schwartz & Buckwalter, 1962) which, although having a larger ring structure than penicillin G may diffuse more rapidly because it has a greatly reduced side chain. Thus, any decrease in diffusion coefficient in the presence of phospholipids would appear not to be due to breakdown products.

Electrolyte addition to the antibiotic solutions gives rise to decreased diffusion coefficients (Table 1). In the absence of electrolyte the "relaxation effect" (Stigter & others, 1955) gives rise to an observed increased diffusion coefficient (D_1) for the benzylpenicillanate anion. Stigter & others (1955) consider this effect to be due to the lagging ionic atmosphere retarding the mobility of the ion. Thus in the case of the slow-moving benzyl penicillanate anion, the presence of its fast-moving counterion (K^+) will result in an increased diffusion coefficient, since, in basic terms, the K^+ ion tends to "pull" the anion from the capillary. Thus the diffusion coefficient (D_2, decreased

relative to D_1), measured in the presence of a swamping concentration of electrolyte in the capillary tube and in the bath, should be the true self-diffusion coefficient (Table 1). Similar considerations apply to ampicillin-electrolyte systems. Neglect of relaxation effects leads to errors in calculations of the fraction bound (Stigter & others, 1955).

Preliminary experiments indicated no necessity for the presence of phospholipid in the bath solution when it was present in the capillary tube; this agrees with the conclusions of Castleden & Fleming (1966), as the diffusion coefficients of the phospholipid aggregates are of the order of 2×10^{-11} m^2s^{-1} (Gammack, Perrin & Saunders, 1964) to $8 \times 10^{-11}m^2s^{-1}$ (Saunders, Perrin & Gammack, 1962). With the exception of lysophosphatidylcholine, the phospholipids reduced the diffusion coefficient of penicillin G to a greater extent than that of ampicillin (Table 1).

A test of residual mean squares (rms) within the data sets was undertaken to confirm no large variability; the ratio of maximum rms to minimum rms may be used to demonstrate whether a significant difference exists between and within the data (Biometrika Statistical Tables, p. 202, Table 31). For 5 data sets with 15 degrees of freedom at 0·95 level, the ratio can be up to 4·37 before the data becomes non-significant. Table 1 shows that in no case was this value obtained, neither between nor within data sets.

The decrease observed for penicillin systems could be due to several effects: the antibiotic could bind to the phospholipid and thus diffuse at a slower rate, with a reduction in the amount of antibiotic available for self-diffusion; because of the presence of large phospholipid aggregates or micelles (less than 0·1 μm diameter) the diffusion path which the antibiotic has to follow will be longer and more tortuous; or the viscosity of the antibiotic-phospholipid solution retards the diffusion of the antibiotic. The latter is unlikely because a 2% dispersion of phosphatidylcholine in water at 25° has a relative viscosity of 1·2202 (Padfield & Kellaway, 1973b). Dispersions used in these studies had much lower concentrations, and hence viscosities.

The decreased diffusion coefficients for both antibiotics is possibly a result of the former effects; other studies (Padfield & Kellaway, 1973a) indicate binding to phospholipids, the greatest effects being observed with penicillin G. The most important phospholipid, as far as the bacterial cell is concerned, is phosphatidylethanolamine (Padfield, 1972), and a considerable reduction in the diffusion coefficient of penicillin G was noted in the presence of this phospholipid (i.e. interaction was occurring), whilst only a small reduction in the value was observed for ampicillin, indicating negligible interaction. The observation that lysophosphatidylcholine reduced the diffusion coefficient of ampicillin to a greater extent than that of penicillin G may be due to the presence of the 2-hydroxyl group in this phospholipid, which may preferentially hydrogen bond to the α-amino group of ampicillin; this effect has also been observed in other work (Padfield, 1972; Padfield & Kellaway, 1973).

The percentages of the antibiotics bound to phosphatidylcholine, lysophosphosphatidylcholine and phosphatidylethanolamine respectively, calculated according to the equation of Castleden & Fleming (1966), are 13·3, 5·2, 9·8 and 10·1, 32·1 and 0% for penicillin G and ampicillin respectively. These values are not in agreement with the percentage bound determined by Sephadex gel filtration (Padfield & Kellaway, 1973a) or equilibrium dialysis (Table 2). These techniques would be expected to give more accurate determinations of the amount bound, as Castleden and Fleming's equations rely solely on differences in diffusion coefficients, which may not be due only to binding of the molecule. The differences between the phospholipids are not as large as observed by other techniques, since the differences in interaction between

Table 2. *Equilibrium dialysis determination of mole % binding of antibiotics to phospholipids.*

	PC	PE	PI	PS	PC and PE as in Table 2.
Penicillin G	39·4	41·6	57·8	4·03	PI = Phosphatidylinositol.
Ampicillin	15·6	18·2	34·6	23·0	PS = Phosphatidylserine.

phospholipids may not be of sufficient magnitude, or of the appropriate type, to produce large changes in diffusion.

Castleden & Fleming (1966, 1970) have observed decreased diffusion coefficients of $^{22}Na^{+}$ in sodium chloride in the presence of albumin and phospholipid sols; they concluded that the decreases were due to electrostatic effects of the macromolecules rather than to binding of the sodium ion.

The diffusion of drugs to their site of action and the binding with biological macromolecules are factors which greatly influence drug disposition and activity. We suggest that a possible explanation for the decreased Gram-negative activity of penicillin G is that it is bound to the large amount of cellular lipid to a greater extent than ampicillin and is prevented from passing to its site of action at the membrane. The binding characteristics have been confirmed (Padfield, 1972), but diffusion through the lipid may be a further contributory factor.

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