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THE BINDING OF THREE PENICILLINS IN THE PLASMA OF SEVERAL MAMMALIAN SPECIES AS STUDIED BY ULTRAFILTRATION AT BODY TEMPERATURE

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The importance of assessing the extent to which the various penicillins are bound to plasma proteins has been emphasized by Bond, Lightbown, Barber & Waterworth (1963). No study of penicillin binding in plasma at body temperature has been reported although there is evidence that the extent of binding varies with temperature (Klotz, Urquhart & Weber, 1950). Most studies to date have been made in man and dog. Values in the dog were lower than those in man. Hence it was felt important to study variations in binding between a number of species and to determine whether the plasma of any species approached human plasma in its ability to bind penicillin. It was also hoped to study some of the factors which may have contributed to the variability of results from different studies. Estimates of binding in dog plasma range from 23% (Anderson, Lee, Worth & Chen, 1956) to 44% (Verwey & Williams, 1962) for benzylpenicillin; from 42% (Pindell, Tisch, Hoekstra & Reiffenstein, 1960) to 61% (Verwey & Williams, 1962) for phenethicillin and from 34% (Anderson *et al.*, 1956) to 64% (Verwey & Williams, 1962) for phenoxymethylpenicillin.

Ultrafiltration was preferred to equilibrium dialysis because: penicillins in plasma at body temperature are unstable over the period required for equilibrium dialysis whereas ultrafiltration can be completed in 15 min; when plasma is dialysed against a buffer the plasma is diluted and its ionic composition changed by influx of water and ions from the buffer; using ultrafiltration any particular concentration of penicillin desired can be obtained in the plasma.

METHODS

Assay

Penicillins were assayed by the cup-plate method using *Sarcina lutea*. The regression of (zone diameter)^2 on log dose consistently gave fiducial limits of less than ± 5% at $P=0.95$. The presence of plasma reduced the zone diameters but the relationship was still linear. Thus in every assay control plasma from the same animal was added to all solutions (plasma, ultrafiltrate and standard) to give the same total plasma concentration in each.

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Ultrafiltration (see Fig. 1)

A 14 in. length of 0.25 in. Visking tubing (F) was washed in distilled water and tied round a piece of silicone rubber tubing (O) at the end of a glass tube (N). A silicone rubber bung (K) was tied into the end of the Visking tubing which was then inflated, and N was closed. The assembly was autoclaved in a dressing sterilizer for 15 min at 15 lb/in.^{2} A vacuum was then drawn in the sterilizer and this dried the tubing in its inflated state. Thus no error was introduced into the subsequent ultrafiltration by the presence of water in the tubing. A wick (G) drawing water from a test-tube (J) was suspended from the other tube (N). In the centre of the wick was a thermocouple (H). The assembly was placed in a sterile column (B). Water at 37° C was circulated through the various jackets surrounding B, the collecting tube (C) and the reservoir (A). The column was evacuated by Hi-Vac pump connected to E, and E was then closed. During evacuation evaporation of water caused a fall in the temperature of the wick as measured by the thermocouple. When the temperature had returned to 37° C it was assumed that evaporation had ceased, that the air in the column was once again saturated and that the system was in equilibrium. After a further 30 min, 30 ml. of plasma, to which the required amount of penicillin had been added, was run into the Visking tube from the reservoir A. Smaller amounts of plasma could be used depending on the volume of ultrafiltrate required and the proportion of the original volume one was willing to take as ultrafiltrate (see below). 2 ml. of ultrafiltrate were produced in about 15 min. Samples of the original plasma and the ultrafiltrate were taken for assay. The ultrafiltrate was tested for protein.

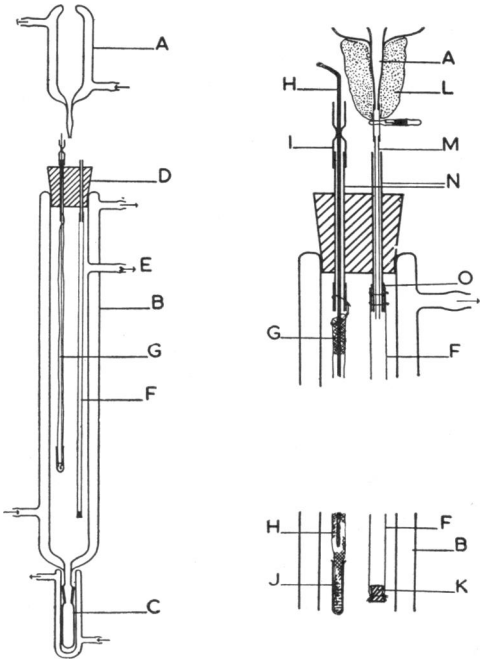


Fig. 1. Ultrafiltration apparatus. For explanation see text.

Solutions of each of the penicillins in 0.15 M-phosphate buffer were ultrafiltered. In no experiment did the concentration of penicillin and sodium in the ultrafiltrate differ significantly from that in the original solution. This ensured that there was no adsorption to or filtering out by the membrane and that there was no evaporation of ultrafiltrate.

Estimation of sodium and chloride

Sodium was measured in an EEL flame photometer, and chloride by the method of van Slyke (1923).

Plasma

Blood was collected into centrifuge bottles containing 2 U of heparin per ml. of blood. It was then centrifuged at 3,000 revs/min for 15 min and the plasma drawn off. Penicillin was added at the desired concentration. Heparin did not affect the binding as shown by the fact that similar results were also obtained when oxalate was used as anticoagulant. Neither did heparin at the concentration used displace penicillin from a 4% solution of bovine albumin in buffer.

Penicillins

Benzylpenicillin (Penicillin G; Glaxo Laboratories), phenoxymethylpenicillin [Penicillin V; Distillers Co. (Biochemicals)] and phenethicillin (Broxil; Beecham Research Laboratories) were used.

Animals

Blood was obtained from animals of the following species: goat (two cross-bred females), pig (two Landrace gilts), ox (a Friesian cow and heifer), horse (a hunter mare and a pony gelding) and sheep (two Border Leicester ewes).

RESULTS

Effect of the plasma becoming concentrated during ultrafiltration

As ultrafiltration proceeds the concentration of protein in the plasma increases. Toribara, Terepka & Dewey (1957) have pointed out that this should not change the concentration of free drug if binding obeys the law of mass action because: $Drug_{free} = (K \cdot Protein_{bound})/Protein_{free}$ and the ratio $Protein_{bound}/Protein_{free}$ remains constant as ultrafiltration proceeds. (This condition is not fulfilled when the proteins are diluted during dialysis.) However, during concentration of the protein other changes, for instance in the Donnan distribution, might upset this equilibrium. Table 1 shows the results of an experiment in which serial samples of ultrafiltrate were taken during ultrafiltration.

TABLE 1

THE CONCENTRATION OF BENZYLPENICILLIN IN SERIAL SAMPLES OF THE ULTRAFILTRATE FROM 30 ML. OF PLASMA

$Penicillin_{uf}$ = penicillin in ultrafiltrate

Volume ultrafiltered (ml.)	$Penicillin_u$ (μg/ml.)
1·5	5·87
4·6	5·72
7·8	5·93
10·0	5·55
14·1	5·47
17·3	5·55
19·8	5·52

Donnan distribution

The penicillins used have pK_A's of 2.7 (Rapson & Bird, 1963) and thus, being ionized at plasma *p*H, will be distributed across the Visking membrane in accordance with the Donnan equilibrium. To assess the Donnan effect sodium and chloride were measured in the ultrafiltrate and plasma. The results are shown in Table 2. Because of the space occupied by the plasma proteins the concentration of an ion in plasma water exceeds the concentration in whole plasma. Plasma water may be calculated as [99.6 – 0.75

TABLE 2

THE RATIOS OF SODIUM AND CHLORIDE CONCENTRATIONS IN ULTRAFILTRATE TO THOSE IN THE PLASMA

Numbers of experiments are in parentheses. Values are means and standard errors

Ratio	Concentration ratio for Sodium (9)	Chloride (7)
Ultrafiltrate: plasma	0·988±0·0044	1·121±0·0112
Ultrafiltrate: plasma water (by calculation)	0·932	1·057
Plasma water: ultrafiltrate (by calculation)	1·073	0·946

TABLE 3

BOUND BENZYLPENICILLIN IN GOAT PLASMA AS A PERCENTAGE OF THE TOTAL PRESENT

Numbers of experiments are in parentheses. Values are means and standard errors

	Bound benzylpenicillin (%) for concentration 0·1 μg/ml.	1·0 μg/ml.	10·0 μg/ml.
Goat 1	52·9±0·7 (2)	53·2±0·1 (2)	52·3±0·3 (3)
Goat 2	55·4±2·0 (3)	52·8±1·9 (3)	52·3±1·4 (4)

TABLE 4

PERCENTAGE OF PENICILLINS BOUND IN THE PLASMA OF VARIOUS SPECIES

Number of experiments are in parentheses

Species	Percentage bound in plasma of Phenethicillin	Phenoxymethylpenicillin	Benzylpenicillin
Horse	—	68·2±0·6 (2)	62·1±0·7 (2)
Goat	71·7±1·4 (4)	66·2±0·4 (3)	52·3±0·8 (7)
Ox	65·9±0·4 (2)	63·1±0·1 (2)	48·3±0·8 (4)
Sheep	60·0±0·5 (2)	58·8±1·0 (2)	42·2±2·2 (2)
Pig	59·7±1·4 (2)	55·8±1·3 (2)	36·6±0·9 (2)

(% protein)] ml. per 100 ml. plasma (McLean & Hastings, 1935). The protein content of goat plasma was 7% and so concentrations in plasma were multiplied by a factor of 1.060 to convert them to concentration in plasma water. The results in Table 2 come close to fulfilling the requirements of the Donnan Equilibrium which states that $Na^+_{uf}/Na^+_{pw} = Cl^-_{pw}/Cl_{uf}$, where pw is plasma water and uf is ultrafiltrate. Scatchard, Scheinberg and Armstrong (1950) have shown that chloride is bound in plasma whereas sodium is not. This probably accounts for the different plasma: ultrafiltrate ratios for sodium and chloride and indicates that sodium is the more reliable measure of the distribution.

Thus $\text{Penicillin-free}_{pw}/\text{Penicillin}_{uf} = Na^+_{uf}/Na^+_{pw} = 0.932$.

Therefore, $\text{Penicillin-free}_{wp} = \text{Penicillin}_{uf} \times 0.932/1.060 = \text{Penicillin}_{uf} \times 0.879$.

Hence percentage binding is $100 \times (\text{Penicillin}_{wp} - 0.879\ \text{Penicillin}_{uf})/\text{Penicillin}_{wp}$, where wp is whole plasma.

Binding of benzylpenicillin in goat plasma

The percentage binding of benzylpenicillin was determined in the plasma of two goats at three concentrations covering the therapeutic range. The results are shown in Table 3. Analysis of variance failed to demonstrate a significant difference in binding either between goats or between penicillin concentrations.

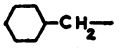
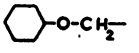
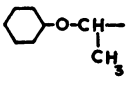
In two experiments whole heparinized blood was used. The percentage binding (mean and standard error) expressed in terms of plasma concentration was $52.0 \pm 0.1\%$. This value does not differ significantly from that obtained with plasma. On four occasions goats were given intramuscular injections of benzylpenicillin. After 20 min blood was taken and the plasma was prepared and ultrafiltered in the usual way. Mean percentage binding was 52.1 ± 1.4. Thus when penicillin is added *in vitro* it is bound to the same extent as *in vivo*.

Binding of three penicillins in various species in vitro

The percentage bindings of benzylpenicillin, phenoxymethylpenicillin and phenethicillin were determined in the plasma of goat, ox, sheep, horse and pig. All penicillins were added at a concentration of 10 μg/ml. The results are shown in Table 4.

TABLE 5

SIDE-CHAINS AND MOLECULAR WEIGHTS OF PENICILLINS

Penicillin	Side-chain	Molecular weight of anion
Benzylpenicillin		333
Phenoxymethylpenicillin		349
Phenethicillin		363

DISCUSSION

Because of the Donnan distribution and the space occupied by the plasma proteins the concentration of penicillin in a plasma ultrafiltrate or dialysate (and presumably also in tissue fluid) is about 10% higher than the concentration of free penicillin in the plasma. For a cationic drug, however, as shown in Table 2, the two concentrations will be similar. Failure to allow for these effects leads to an underestimate of the binding of an anion.

Over the range (albeit 100-fold) of therapeutic concentrations the percentage binding of the penicillins was constant (Table 3), as predicted by Goldstein (1949). Within this range percentage binding for a particular penicillin can therefore be expressed by a single figure. However, percentage binding decreases as penicillin concentration is further increased. Ultrafiltration of whole blood gave the same estimate of binding as ultrafiltration of plasma. This result does not indicate how much penicillin there is in, or on, the red cells but it shows that estimates of the levels of free penicillin in plasma are a valid measure of the concentration of free penicillin in whole blood. The finding that penicillin added to plasma *in vitro* was bound to the same extent as penicillin injected into the animal further confirms the validity of the method.

Table 4 shows the same order of binding, both between species and between penicillins. This suggests that binding is to similar sites in each instance.

The penicillins were bound in the descending order phenethicillin—phenoxymethylpenicillin—benzylpenicillin, suggesting that binding increases with increasing complexity of the side-chain and with increase of molecular weight (Table 5).

Increase of binding with increase of molecular weight has also been found in other series of homologous compounds, for example the barbiturates (Goldbaum & Smith, 1954), the fatty acids (Teresi & Luck, 1952) and the early penicillins (Tompsett, Shultz & McDermott, 1947). It seems likely, therefore, that binding is reinforced by van der Waal's forces.

Horse plasma bound the highest percentage of the penicillins while goat, ox, sheep and pig plasmas bound progressively less. The penicillins, like most anions, are bound almost exclusively to albumin (Tompsett *et al.*, 1947). Thus factors which may vary between species and account for species differences in binding are the concentration of albumin, its affinity for penicillin and the presence in plasma of substances which compete with penicillin for the binding sites.

The average plasma concentrations of albumin, expressed relative to man, are goat, 0.90 ; pig, 0.88 ; horse and ox, 0.72 ; and sheep, 0.70 (Spector, 1956). Thus, as Genazzani & Pagnini (1963) pointed out when discussing the binding of sulphonamides, differences in albumin concentration may partly explain the high degree of binding by human plasma but they do not account for the differences between the other species. The fact that different species bind penicillins and sulphonamides in a different order and that there are even differences between individual sulphonamides (Genazzani & Pagnini, 1963) also suggests that variations in albumin concentration are not the sole cause of the species differences.

Species differences in binding ability would also arise if the several albumins had different affinities for the penicillin molecule. No major differences have been found in the composition of the albumins of man, ox, horse and sheep (Sǒrm, 1958). Moreover, Tanford, Swanson & Shore (1955) found that the titration curves of human and bovine albumin were essentially similar. However, the work of Tompsett *et al.* (1947) suggests that human albumin has a greater affinity for benzylpenicillin than has bovine albumin.

A third possible source of species variation is the ionic constitution of the different plasmas and the presence in plasma of competing substances. For instance, long-chain fatty acids displace penicillin from its binding to albumin (unpublished observation) and may be important, especially in ruminants.

Previous estimates of the extent of which penicillins are bound in horse and ox plasma (Acred, Brown, Turner & Wright, 1961 ; Acred, Brown, Turner & Wilson, 1962) were lower than those reported here. Some of the factors which may account for this discrepancy are: (1) The measurements reported here were carried out at body temperature. The effect of this factor is uncertain. Klotz *et al.* (1950) found that a rise of temperature increased binding at low concentrations of penicillin but inhibited binding at higher concentrations. The binding of calcium increases 0.5% for each ° C rise in temperature (Toribara *et al.*, 1957). Anionic dyes, on the other hand, are bound to a lesser degree at higher temperatures (Klotz & Urquhart, 1949), while the binding

of sulphonamides (Davis, 1943) and barbiturates (Goldbaum & Smith, 1954) is unaffected by temperature. (2) Failure to calculate for the effects of the Donnan distribution and of the space occupied by the proteins will underestimate the binding of an anion. (3) Studies carried out at drug concentrations above the therapeutic range will also underestimate the extent of binding. (4) In dialysis studies dilution of protein and change of ionic constitution may alter binding. (5) In the studies reported here and also in those of Acred and his co-workers binding may have been altered by the change in plasma *p*H which is caused by loss of carbon dioxide.

To what extent does binding affect the activity of a penicillin in the body? The concentration of penicillin in lymph (Verwey & Williams, 1962) and in milk (Rasmussen, 1959) is determined by the concentration of free penicillin in the plasma. Levels of penicillin in the brain, although they may be reduced by protein-binding, are probably also limited by a mechanism which actively transports penicillin out of the central nervous system (Pappenheimer, Heisey & Jordan, 1961). It has been pointed out that during the inflammation of any extravascular site the permeability of the capillaries increases and with it the concentration of penicillin at that site. However, this increase is presumably caused by the influx of plasma protein with its attached penicillin and unless the bacterium is able "capture" the penicillin, that is unless it has a higher affinity for penicillin than the receptors on the albumin molecule, it will still be exposed to free penicillin only. Quinn, Colville, Ballard, Jones & Debnam (1962), using a series of penicillins, have shown that bacteria *in vitro* are unable to take up penicillin from plasma proteins. Thus the antibacterial effect of a penicillin in plasma is that of the free penicillin. Qualitatively similar results have been obtained by Barber & Waterworth (1964), and by Bond *et al.* (1963). In view of these findings Bond and her co-workers suggested that penicillin concentrations in the body were best indicated by the concentration of free antibiotic present in the plasma. A crucial test of the importance of binding to plasma proteins would appear to be whether the differences between the antibacterial activities as measured *in vitro* and *in vivo* for each of a series of penicillins correlates with the extent to which they are bound. Gourevitch, Hunt & Lein (1960) showed that this was so. They tested penicillins against the same strain of staphylococcus *in vitro* and *in vivo*. Activity *in vitro* in serum showed a closer correlation with activity *in vivo* than was shown by activity *in vitro* in ordinary media.

SUMMARY

1. An ultrafiltration apparatus is described for measuring the binding of penicillins in plasma at body temperature.

2. The increase in concentration of the plasma proteins which occurred during ultrafiltration did not affect binding. Because of the Donnan effect, as indicated by the distribution of sodium, the concentration of penicillin in the ultrafiltrate was 10% higher than the concentration of free penicillin in the plasma. Within the therapeutic range the percentage of penicillin bound was independent of penicillin concentration.

3. The binding of benzylpenicillin, phenoxymethylpenicillin and phenethicillin in the plasma of goat, ox, sheep, horse and pig was determined. In each of these species the penicillins were bound in the descending order phenethicillin—phenoxymethlypenicillin—benzylpenicillin, while the different species bound each penicillin in the descending order horse—goat—ox—sheep—pig.

4. The reasons for species differences in binding and the effect of protein binding on the *in vivo* activity of penicillins are discussed.

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