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THE BINDING OF ANTIBIOTICS TO SERUM PROTEINS

BY

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The published work on the binding of antibiotics to serum proteins is extensive. Nevertheless, data are still incomplete in certain respects. For example, there is little information on the rate at which antibiotics are bound to the proteins and the rate at which this complex breaks down. Similarly, although the binding of antibiotics to serum proteins is known to be essentially reversible it is not certain whether there is any proportion of the drug which is bound irreversibly. On certain aspects, published data are available but are conflicting and some lack of agreement exists on the precise extent of binding of certain antibiotics, particularly of tetracyclines. There is also a general lack of comparative results on the extent of binding for related antibiotics obtained from experiments carried out by the same method.

In the present paper, results are given for the effect of certain factors on the binding of penicillins and other antibiotics in serum and comparative data are also given for the extent of binding in human serum for all the penicillins at present in clinical use.

METHODS

Measurement of binding of antibiotics to serum proteins

The extent of binding of penicillins and other antibiotics to the proteins of serum was measured by ultrafiltration through Visking viscose-cellulose dialysis tubing. Preliminary experiments indicated that there was no significant difference between the extent of binding in plasma obtained from heparinized blood and that in serum. The antibiotic present in the protein-free ultrafiltrate was measured by microbiological assay and this quantity represented the free, unbound, fraction of antibiotic in serum. The amount of antibiotic bound to protein was derived by subtracting the level of free antibiotic from the known total concentration in serum. Before comparative experiments were made to measure the extent to which different antibiotics were bound to the proteins of human serum, preliminary investigations were carried out into the effects of various experimental factors thought likely to influence the extent of binding. These included the effects of antibiotic concentration, nature of protein, temperature, individual variation, rate of binding and nature of binding.

Ultrafiltraton techniques

Suitable lengths of Visking tubing (0.25 in. internal diameter) were knotted at one end and attached at the other to a manifold connected to compressed air. Serum samples were introduced into the tubing before connexion to the manifold and ultrafiltration was then carried out at room temperature at a positive pressure of 15 lb/in^2. The ultrafiltrate was collected in a glass tube which closely surrounded the Visking sac. In most experiments the volume of serum used was 5 ml., and the volume of ultrafiltrate collected for assay was about 0.5 ml. In some experiments a volume of 10 to 12 ml. of serum was used which permitted an adequate sample of ultrafiltrate to be collected in about 7 min.

Horse, sheep, rabbit, and calf sera were obtained as commercial samples from Burroughs Wellcome. Human serum was obtained from healthy volunteers and pooled before use. The *p*H of the fresh human serum was approximately 7.4, but on standing in the refrigerator for a few days the *p*H rose to as high as 8.0 or above. Because of this change in *p*H, in all the experiments the *p*H of the serum was adjusted to 7.4 with carbon dioxide before use, although in a limited number of experiments with benzylpenicillin and cloxacillin the extent of binding did not appear to be greatly influenced by changes in *p*H over the range 7.4 to 8.2.

With each antibiotic it was confirmed that there was free passage of the compound through the membrane without selective filtration when aqueous solutions were placed inside the Visking tube and air pressure was applied. It was also shown that the ultrafiltrate from normal serum was devoid of protein. There was no evidence that blocking of the membrane took place during filtration, and in repeat experiments with different penicillins consecutive samples of ultrafiltrate showed no significant difference in assay results. In contrast, with the tetracyclines there was some evidence that the concentration in the first sample of ultrafiltrate was erroneously low and a subsequent sample was routinely used for assay. This phenomenon with tetracycline has already been reported by Remington & Finland (1962).

Measurement of free and total antibiotic

The antibiotic content of samples of serum or serum ultrafiltrate was determined by microbiological assay. Rectangular glass plates (12 × 15 in.) were poured with a layer of nutrient agar (Oxoid No. 2) inoculated with a suspension of a suitable assay organism. For assay of penicillins and cephalothin, *Bacillus subtilis* (N.C.T.C. 8236) or *Sarcina lutea* (N.C.T.C. 8340) was employed. The tetracyclines were assayed with *Bacillus cereus* (N.C.TC 8035), and novobiocin with the Oxford strain of *Staphylococcus aureus* (N.C.T.C. 6571). Holes (7 mm) were punched in the seeded agar with a No. 4 cork-borer, and these were filled with the test samples or appropriate standard solutions.

For estimation of free concentrations of penicillins in ultrafiltrate samples, standard solutions were prepared in 0.05 M-phosphate buffer (*p*H 7.4), preliminary experiments having shown that standard penicillin solutions in phosphate buffer yielded results identical to equivalent standard solutions prepared in ultrafiltrate from normal human serum. With the tetracycline antibiotics, however, preliminary experiments showed that standard solutions in buffer produced inhibition zones which were significantly larger than those caused by the equivalent solutions prepared in serum ultrafiltrate. Accordingly, with this group of antibiotics, and with novobiocin and fusidic acid, standard solutions were prepared in normal serum ultrafiltrate. Total antibiotic concentrations in serum were measured against standard solutions prepared in normal human serum, *p*H 7.4. Inhibition zone diameters were measured after incubation overnight at 30° C and the concentrations of antibiotic were estimated by reading from a graph derived from similar assays of standard solutions.

Determination of minimum inhibitory concentrations

Minimum inhibitory concentrations required to prevent growth of bacteria for 24 hr at 37° C were determined by serial dilution of the antibiotic in 1 ml. volumes of nutrient broth (Oxoid No. 2). In addition, minimum inhibitory concentrations were also determined in serum in the same fashion by serial dilution in 1 ml. volumes of normal human serum, *p*H 7.4. For the determinations of minimum inhibitory concentrations in serum, aqueous solutions of the antibiotic were added to give the desired range of antibiotic concentrations with a final serum concentration of 95%. One drop of a 1/1,000 dilution of an overnight culture was added to each tube to give an inoculum of about 10^4 organisms. Visual determination of minimum inhibitory concentrations in serum was not always definite, and with the penicillins the end points were checked by subculturing from the serial dilution onto antibiotic-free agar, and observing growth after a further 24 hr incubation.

RESULTS

Factors affecting the binding of antibiotics to serum proteins

Concentration of antibiotic. Fig. 1 illustrates the effects of variation of antibiotic concentration on the extent of binding of certain penicillins in human serum. With

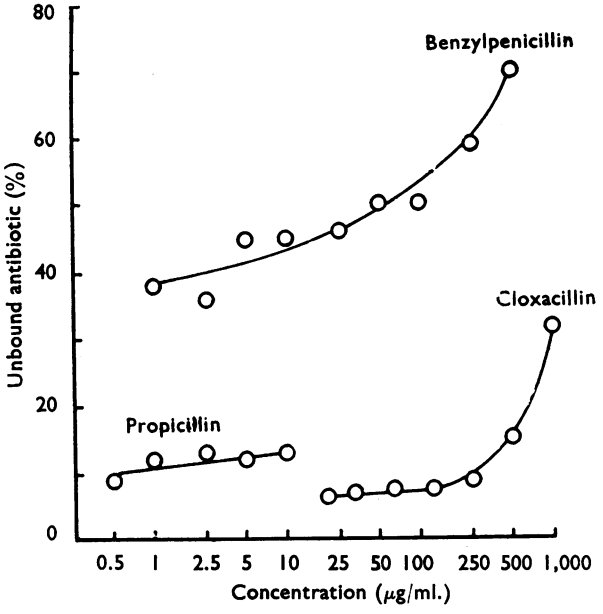

Fig. 1. Effect of variation in the concentration of penicillin on the extent of binding in serum. Human serum containing various concentrations of benzylpenicillin, propicillin or cloxacillin was filtered through Visking membranes. The protein-free ultrafiltrates, containing the unbound compound, were assayed for antibiotic content.

benzylpenicillin and cloxacillin the percentage of unbound drug in serum increased greatly with increase in concentration above approximately 200 μg/ml. of penicillin. Below this value changes in concentration appear to have a relatively slight effect on the extent of binding.

TABLE 1

EXTENT OF BINDING OF BENZYLPENICILLIN AND CLOXACILLIN IN SERA OF DIFFERENT ANIMAL SPECIES

Sera containing benzylpenicillin (1 μg/ml.) or cloxacillin (50 μg/ml.) were filtered through Visking membranes. The ultrafiltrates, containing the free, unbound penicillin of each serum specimen, were assayed for antibiotic content. Unbound penicillin was calculated as the ratio of the concentration in ultrafiltrate to the total concentration in serum, × 100

	Unbound penicillin (%) for	
Species	Benzylpenicillin	Cloxacillin
Man	49·0	6·5
Horse	59·0	30·0
Sheep	70·0	19·5
Rabbit	65·0	22·0
Calf	63·0	25·0

Nature of protein. Table 1 shows the extent of binding of benzylpenicillin and cloxacillin to sera of different animal species. The extent of binding of a particular penicillin to serum may differ greatly from one species to another, the results with cloxacillin in human and horse serum being particularly marked. Thus, in horse serum the unbound proportion of cloxacillin was about five-times greater than in human serum.

With both benzylpenicillin and cloxacillin the degree of binding was greatest with human serum, but the relative binding capacities of the other sera were not the same for both penicillins.

Concentration of protein. The relationship between concentration of human serum and extent of binding is shown in Fig. 2 for benzylpenicillin and cloxacillin. Human serum was diluted to the appropriate concentration with distilled water and the determinations

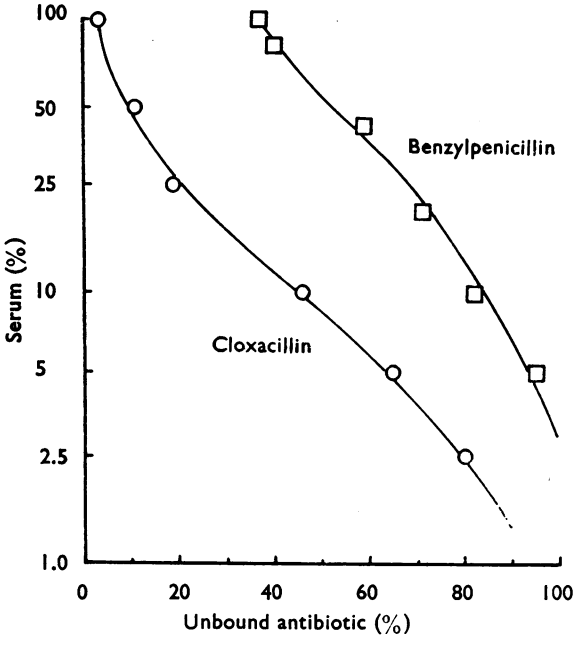

Fig. 2. Effect of variation in the concentration of serum protein on the extent of binding of benzylpenicillin and cloxacillin. Human serum was diluted with 0.05 M-phosphate buffer (*p*H 7.4) to give solutions of various protein concentrations. These solutions containing benzylpenicillin (1 μg/ml.) or cloxacillin (50 μg/ml.) were filtered through Visking membranes and the protein-free ultrafiltrates, containing the unbound penicillin of each solution, were assayed for antibiotic content.

of extent of binding were made by ultrafiltration of samples of aqueous sera containing benzylpenicillin at 2 μg/ml. and cloxacillin at 50 μg/ml. of serum. As would be expected, the extent of binding of both penicillins decreased as the concentration of protein was reduced, and the relative increase in unbound antibiotic with decreasing serum concentration was very similar for benzylpenicillin and cloxacillin. Nevertheless, with cloxacillin only about 50% of the penicillin was available as unbound antibiotic even after a ten-fold dilution of the serum.

Temperature. The percentage binding of benzylpenicillin and cloxacillin in human serum was determined by ultrafiltration at 37, 22 and 4° C. Solutions of benzylpenicillin (1 μg/ml. of serum) and cloxacillin (50 μg/ml. of serum) were adjusted to the required temperature before filtration and the experiments were carried out in temperature controlled rooms. The temperatures of the samples of ultrafiltrate were checked as they

were collected and did not differ significantly from the initial temperatures. In several experiments with benzylpenicillin and cloxacillin, variation in temperature had little effect on the extent of binding of the penicillins, and with both compounds results obtained at 37° C differed little from those obtained at 4° C.

Binding in sera of individual human subjects. Serum was obtained from each of six healthy subjects. Benzylpenicillin was added to give a concentration of 2 μg/ml. and the extent of binding was determined by ultrafiltration. Ultrafiltration was repeated the following day using a further aliquot of each of the samples of sera. One week later serum was again obtained from the same six subjects and the determination of binding with benzylpenicillin was carried out on two successive days as before. The results are shown in Table 2. Differences in binding capacity between one subject and another were slight and might well be less than the experimental errors inherent in the methods. Further

TABLE 2

VARIATION IN THE EXTENT OF BINDING OF BENZYLPENICILLIN IN SERA OF INDIVIDUAL HUMAN SUBJECTS

Serum was obtained from each of six normal subjects and benzylpenicillin added to give a concentration of 2 μg/ml. The extent of binding was determined by ultrafiltration and the experiment was repeated the following day using a further aliquot of each serum sample (Experiment 1). One week later serum was again obtained from the same six subjects and the determination of binding carried out on two successive days as before (Experiment 2)

	Unbound antibiotic (%) in			
	Experiment 1		Experiment 2	
Subject	First determination	Repeat determination	First determination	Repeat determination
1	47·5	41·0	39·0	36·5
2	47·5	46·0	41·0	39·5
3	49·0	49·0	40·0	40·0
4	57·0	49·5	42·0	40·0
5	49·0	44·5	33·5	35·0
6	50·0	41·5	39·0	40·5

experiments with another group of six subjects using both benzylpenicillin and cloxacillin confirmed these results and failed to indicate marked differences in binding capacity between one person and another.

Rate of binding and dissociation of penicillin in human serum. Experiments were carried out with benzylpenicillin, phenoxymethylpenicillin, phenethicillin, methicillin, cloxacillin and ampicillin to determine the rate at which binding takes place in human serum. Ultrafiltration was carried out immediately after the addition of the penicillin to the serum and further samples were also taken at intervals of time up to 1 hr. The time taken to obtain the first sample of ultrafiltrate was about 8 min after adding the penicillin to the serum. With all the penicillins tested the assay of the initial sample did not differ significantly from any of the subsequent samples indicating that equilibrium had been reached by the time the first sample had been collected.

The following experiments were also carried out to determine the rate of breakdown of the penicillin-protein complex. Cloxacillin was dissolved in serum to give a penicillin concentration of 400 μg/ml. and a sample of ultrafiltrate was obtained. At this concentration 13% of the penicillin was present in the serum as free, unbound, cloxacillin. The

serum was then diluted fortyfold with 0.05 M-phosphate buffer (*p*H 7.4) to give a serum concentration of 2.5%, and samples of ultrafiltrate were taken immediately after dilution and also at intervals of time up to 1 hr. Assay of the sample taken immediately after dilution showed 49% unbound antibiotic, and assays of the subsequent samples showed that the new equilibrium had been established by the time the first sample was taken.

Extent of irreversible binding in serum. Cloxacillin was dissolved in human serum at a concentration of 400 μg/ml. and a sample of ultrafiltrate was obtained in the usual way. Under these conditions 87% of the antibiotic was bound to protein. An aliquot of the original serum solution was then diluted fortyfold with 0.05 M-phosphate buffer, *p*H 7.4, which resulted in dissociation of the antibiotic-protein complex and yielded a solution consisting of 2.5% serum in buffer and cloxacillin at a concentration of 10 μg/ml. A sample of ultrafiltrate was then obtained and compared with ultrafiltrate from a solution of 10 μg/ml. cloxacillin in 2.5% serum prepared directly by adding cloxacillin to 2.5% serum. Results, typical of those obtained when the experiment was repeated, are shown in Table 3. After dilution of the serum with buffer the concentration of unbound antibiotic was slightly lower than in the solution prepared directly at the same

TABLE 3

THE EXTENT OF IRREVERSIBLE BINDING OF CLOXACILLIN IN HUMAN SERUM

Human serum containing cloxacillin (400 μg/ml.) was diluted fortyfold in 0·05 M-phosphate buffer (*p*H 7·4), and the unbound penicillin present was measured by ultrafiltration. The extent of binding of a solution of cloxacillin (10 μg/ml.) prepared directly in 2·5% human serum was also determined. The amount of irreversible binding of cloxacillin to serum is indicated from a comparison of the amounts of free cloxacillin in the two solutions

Solution	Cloxacillin concentration (μg/ml.)	Serum concentration (%)	Antibiotic	
			Bound (%)	Unbound (%)
Original	400	100	87	13
Diluted fortyfold	10	2·5	49	51
Prepared directly	10	2·5	40	60

serum concentration. Further experiments were also carried out with oxacillin at two concentrations, 400 and 20 μg/ml., before dissociation by dilution with buffer. As in the experiments with cloxacillin the concentration of unbound drug was slightly higher in the solution prepared directly in 2.5% serum than in the corresponding solution obtained by dilution of normal serum with buffer. These results suggest that some degree of irreversible binding may take place, although it appears that this is only a small proportion of the total antibiotic in normal serum.

Competitive binding between cloxacillin and other substances. The results of experiments to determine the extent of binding of cloxacillin in human serum in the presence of substances which are themselves highly bound to serum proteins are shown in Table 4. In these experiments various concentrations of compounds were added to serum containing 50 μg/ml. cloxacillin. Ultrafiltrates of serum containing these compounds alone showed no antibacterial activity which would have obscured the activity of unbound cloxacillin, except in the experiments with novobiocin and sulphonamides. To eliminate activity due to novobiocin the assay of cloxacillin was performed with a novobiocin-resistant strain of *Staph. aureus*; sulphonamide activity was neutralized by the addition of *p*-aminobenzoic acid to the assay agar.

Table 4 shows that the level of unbound cloxacillin in serum was four- to fivefold greater than normal in the presence of 500 μg/ml. of sodium salicylate, γ-resorcylic acid, sulphamethoxypyridazine or phenylbutazone, but that at a concentration of 100 μg/ml. these substances resulted only in a slight increase in the amount of unbound cloxacillin. These results were not influenced by the order in which the cloxacillin was added to the serum,

TABLE 4

COMPETITIVE BINDING BETWEEN CLOXACILLIN AND OTHER SUBSTANCES

Cloxacillin was added, to give a concentration of 50 μg/ml., to aliquots of human serum containing various compounds at concentrations of 100 and 500 μg/ml. The sera were filtered through Visking membranes and the amount of unbound cloxacillin was measured by assay of the ultrafiltrates. Ultrafiltrates of serum containing these compounds alone showed no antibacterial activity which would have obscured the activity of unbound cloxacillin, except in the experiments with novobiocin and sulphonamides. To eliminate activity due to novobiocin the assay of cloxacillin was performed with a novobiocin-resistant strain of *Staph. aureus*; sulphonamide activity was neutralized by the addition of *p*-aminobenzoic acid to the assay agar

	Unbound cloxacillin (%) for concentration of competitive compound	
Competitive compound	100 μg/ml.	500 μg/ml.
None	6·6	6·6
Sodium salicylate	8·8	33·0
γ-Resorcylic acid	10·8	32·0
Sulphamethoxypyridazine	8·0	23·5
Phenylbutazone	10·4	23·0
Sulphadimidine	8·9	20·6
Novobiocin	—	7·0

either before or after the addition of the competitive compound to the serum. The addition of novobiocin at a concentration of 500 μg/ml. to serum containing cloxacillin had no significant effect on the extent of binding of cloxacillin although novobiocin is known to be highly bound to serum proteins.

Measurement of the extent of binding of various antibiotics to the proteins of human serum

Results for the extent of binding in human serum are given in Table 5 for all the penicillins in clinical use at present. Results are also given for cephalothin, tetracycline, chlortetracycline, demethylchlortetracycline, novobiocin and fusidic acid. The determinations of protein binding were carried out by ultrafiltration of human serum, as described in Methods, at room temperature and *p*H 7.4. The concentrations of the antibiotics were governed by the extent of binding of each compound and the sensitivity of the assay but, in general, these concentrations were of the same order as those reached in serum in humans following the recommended dosage schedules. In many instances, determinations were also made at more than one drug concentration, and for any particular antibiotic no significant differences in protein binding could be detected over the ranges of concentration used. Data for groups of closely related antibiotics such as phenoxymethylpenicillin, phenethicillin and propicillin and tetracycline, chlortetracycline and demethylchlortetracycline were obtained in comparative experiments using the same batches of pooled serum, although there was no evidence of variation in binding capacity between one batch of serum and another.

TABLE 5

THE EXTENT OF BINDING OF VARIOUS PENICILLINS AND OTHER ANTIBIOTICS IN HUMAN SERUM

Aliquots of human serum containing the respective antibiotics were subjected to ultrafiltration and the ultrafiltrate was assayed for antibiotic content. The percentage of bound antibiotic in serum was calculated as the difference between the total concentration in serum and the concentration in ultrafiltrate divided by the total concentration in serum, $\times 100$. The concentrations of the antibiotics used were governed by the extent of binding of each compound and the sensitivity of the assay, but in general these concentrations were of the same order as those reached in serum in humans following the recommended dosage schedules

Antibiotic	Concentration in serum (μg/ml.)	Unbound (%)	Bound (%)
Benzylpenicillin	1	41	59
Phenoxymethylpenicillin	5	20·3	79·7
Phenethicillin	10	17·3	82·7
Propicillin	10	11·5	88·5
"Rixapen"	10	8·4	91·6
Phenbenicillin	10	2·8	97·2
Nafcillin	50	13·2	86·8
Oxacillin	50	6·9	93·1
Cloxacillin	50	6·0	94
Methicillin	10	50·7	49·3
Ampicillin	5	82	18
Tetracycline	20	30	70
Chlortetracycline	20	13	87
Demethylchlortetracycline	20	11	89
Novobiocin	100	0·8	99·2
Fusidic acid	50	2·8	97·2
Cephalothin	5	23	77

Among the penicillins, ampicillin was the least affected by the presence of human serum, 82% of the compound being present as free antibiotic. The acid-stable penicillins, phenoxymethylpenicillin and phenethicillin were both bound to about the same extent, the extent of binding being such that the free level of each drug was about half that of benzylpenicillin. Propicillin was bound somewhat more than phenoxymethylpenicillin and phenethicillin while phenbenicillin was bound to a significantly greater extent. Among the group of penicillinase-stable penicillins, nafcillin, oxacillin and cloxacillin were all highly bound to the proteins of human serum, whereas methicillin was bound to a relatively small extent, about 50% of the drug being present in the free form.

Like the penicillins, the tetracycline group of antibiotics showed differences in the relative extent of binding to serum proteins. Both chlortetracycline and demethylchlortetracycline showed a high degree of binding and were bound to a significantly greater extent than was tetracycline. With the cephalosporin derivative, cephalothin, about one-quarter of the compound was present in serum as free antibiotic.

Both novobiocin and fusidic acid were highly bound to the proteins of human serum. In the case of novobiocin, only 0.8% of the drug was available as unbound antibiotic, this being the highest degree of binding of antibiotic to serum measured in this series of experiments.

Effect of serum on antibacterial activity

Results are given in Table 6 for the minimum inhibitory concentrations of benzylpenicillin, phenoxymethylpenicillin, phenethicillin, propicillin and cloxacillin, against *Staph. aureus* (Smith) in nutrient broth and in 95% human serum. These compounds

TABLE 6

CORRELATION BETWEEN THE EXTENT OF BINDING OF VARIOUS PENICILLINS IN HUMAN SERUM WITH THE DIMINUTION OF THE ANTIBACTERIAL ACTIVITY OF THESE COMPOUNDS IN THE PRESENCE OF HUMAN SERUM

Minimum inhibitory concentrations of the penicillins against *Staph. aureus* (Smith) were determined in nutrient broth and in 95% human serum. The percentage of penicillin bound to serum was calculated as the difference between the minimum inhibitory concentration in serum and the minimum inhibitory concentration in nutrient broth, divided by the minimum inhibitory concentration in serum, $\times 100$; this was compared with values obtained by ultrafiltration

Penicillin	No. of expts.	Average minimum inhibitory concentration (μg/ml.)		Extent of binding (% bound) determined	
		Nutrient broth	95% Human serum	From the minimum inhibitory concentration	By ultrafiltration
Benzylpenicillin	2	0·02	0·04	50	59
Phenoxymethylpenicillin	4	0·017	0·067	75	79
Phenethicillin	4	0·045	0·18	76	82
Propicillin	4	0·055	0·36	85	88
Cloxacillin	2	0·21	3·1	93	94

differ markedly with regard to the effect of serum on antibacterial activity. In each instance activity was diminished, but whereas with benzylpenicillin activity was depressed only twofold, with cloxacillin the decrease was approximately fifteenfold. In the presence of serum the proportion of the drug bound to protein is generally believed to be without antibacterial activity (Goldstein, 1949). The percentage of the drug bound can therefore be estimated from the ratio of the activities in the absence and in the presence of serum. These values have been calculated and are shown in Table 6, together with values for the extent of protein binding as determined in the subsequent experiments using ultrafiltration. There is a fairly close correlation between the diminution of activity in serum and the proportion of the drug bound as determined by ultrafiltration.

DISCUSSION

Although some evidence is presented here which suggests that a small proportion of drug may be irreversibly bound in human serum, the phenomenon of binding appears to be essentially reversible. Of the factors which affect the equilibrium between protein and free drug on the one hand and protein-bound antibiotic on the other, the nature of the drug itself and the type of serum appear to be the most important. For example, with different antibiotics the amount of unbound drug in human serum may range from 82% with ampicillin to as low as 0.8% with novobiocin. Similarly, for a given antibiotic the extent of binding in the serum of one animal species may differ greatly from that in another. With cloxacillin, for example, the proportion of unbound drug in human serum was 6.5% compared with 30% in horse serum. Variation in binding capacity for sulphonamides between sera of different animal species has been reported also by Anton (1960). As with the penicillins reported here, binding of the sulphonamides was generally highest in human serum compared with that of other species. It is of some interest that sera from different species appear to have different relative binding capacities for different antibiotics. It is of particular importance, when a number of substances are evaluated experimentally in one animal species, that the assumption is not made that the relative extent of binding of the compounds will be the same in some other species.

Temperature has been reported as a factor influencing the extent of protein binding of antibiotics. Klotz, Urquhart & Weber (1950) reported that the extent of binding of penicillin decreased with increase in temperature and Scholtan & Schmid (1962, 1963) reported the binding of penicillin and tetracyclines to be lower at 37 than at 4° C. On the other hand, Wozniak (1960) reported binding of tetracycline to be slightly greater at 37 than at 2 to 5° C. In the case of sulphonamides, Davis (1943) reported binding to be little influenced by temperature. In the experiments reported here with two penicillins, binding was not significantly influenced by temperature in the range 4 to 37° C.

With regard to the effect of antibiotic concentration on the extent of binding, it is known that the proportion of unbound drug increases with increase in drug concentration (Davis, 1943; Goldstein, 1949). In the case of certain penicillins a significant difference in the extent of binding was reported by Scholtan & Schmid (1962) in a range of concentrations as low as 0.5 to 8.0 μg/ml. On the other hand, from the data given by Kunin (1961) for certain penicillins there appeared to be little effect of concentration in the range 5 to 20 μg/ml., and Verwey & Williams (1962), using dog plasma, reported that variation in penicillin concentration from 5 to 100 μg/ml. did not alter the percentage of the drug bound. In experiments reported here with benzylpenicillin and cloxacillin, the percentage unbound drug increased sharply at concentrations over approximately 200 μg/ml. but at the levels normally achieved in the body during therapy it appears that changes in concentration do not greatly influence the extent of binding. It appears that in serum containing penicillin at concentrations of 200 μg/ml. or greater a significant proportion of the binding sites on the serum proteins are occupied, and consequently with further increase in penicillin concentration the proportion of the drug which can be bound must fall. As is shown in Table 4 the same binding sites can also be occupied by other substances such as sodium salicylate or a sulphonamide and when this occurs the extent of binding of a penicillin, such as cloxacillin, is diminished. These substances, however, must be present at a concentration greater than 100 μg/ml. in order to occupy a significant proportion of the binding sites on the serum proteins and thereby diminish the extent of binding of a penicillin.

Competitive binding in serum between different substances has been described by Anton (1960) who reported displacement of protein-bound sulphonamide with a number of substances including phenylbutazone, and the displacement of plasma-bound bilirubin by sulphonamides is well established (Odell, 1959). More recently, Kunin (1964) has reported competitive binding between penicillins and other substances with results similar to those described here. Competition does not always occur between compounds which are extensively bound to protein; in our experiments, novobiocin had no significant influence on the binding of cloxacillin, presumably because different binding sites were involved.

Bond, Lightbown, Barber & Waterworth (1963) have reported that the extent of binding of penicillin in human serum varied from subject to subject and that this difference was sufficiently marked to make the use of pooled serum unsuitable for the preparation of standards for the measurement of penicillin in the sera of individual subjects. The experiments reported here have failed to confirm this, and suggest that differences in binding capacity between one subject and another are slight and not sufficient to influence signifi-

cantly the assays of serum levels. For all practical purposes the use of normal pooled serum should give reliable results in the measurement of antibiotic activity.

The results in Table 5 showing the extent of binding of different antibiotics in human serum emphasize the great variation which exists between one compound and another. Among the analogues of phenoxymethylpenicillin the differences in binding between phenoxymethylpenicillin and phenethicillin appear to be slight, but with phenbenicillin the binding in serum is significantly greater. With the penicillinase-stable penicillins, binding with cloxacillin is much higher than with methicillin. However, cloxacillin is considerably more active than methicillin and, from a consideration of the total serum levels obtained with the recommended therapeutic doses of these compounds, the activity of the unbound fraction of cloxacillin in serum is at least equal to that obtained with methicillin. With oxacillin the extent of binding is similar to that of cloxacillin but the total serum levels obtained are significantly lower for equivalent dosage (Knudsen, Brown & Rolinson, 1962). Consequently, the levels of unbound oxacillin are also lower than those obtained with cloxacillin.

With the broad-spectrum antibiotics, differences in binding are particularly marked. For example, with ampicillin, 82% of the drug in human serum is free penicillin, whereas with tetracycline only 30% of antibiotic is unbound and with demethylchlortetracycline the corresponding value is as low as 11%. Total serum concentrations obtained with ampicillin are of the same order as those obtained with tetracyclines, and antibacterial activities *in vitro* are also comparable against many pathogens. In such instances the differences in extent of binding of these compounds to the proteins of human serum may be a significant factor in determining therapeutic effect *in vivo*.

Our results relating the activity of certain penicillins in nutrient broth with the activity of these substances in human serum show that there is a fairly close correlation between the diminution of antibacterial activity in serum and the extent to which the drug is bound as determined by ultrafiltration. These observations agree with results already reported by others (Tompsett, Schultz & McDermott, 1947; Colville & Quinn, 1961; Quinn, Colville, Ballard, Jones & Debnam, 1962; Kirby, Rosenfeld & Brodie, 1962) and support the view that the protein-bound drug is inactive (Davis, 1942, 1943; Goldstein, 1949; Goodman & Gilman, 1955; Lambert, 1964), and that the activity in serum is that of the unbound fraction. Direct determination of activity of the protein-bound antibiotic is not possible because dissociation of the antibiotic-protein complex to give an equilibrium between bound and free drug is unavoidable. From the indirect evidence, however, it seems that the activity of the bound drug is negligible. Since the protein-bound drug is essentially inactive and also relatively non-diffusible (Davis, 1943; Goldstein, 1949; Scholtan & Schmid, 1962) the levels of unbound drug obtained in the serum are likely to be more significant than are the levels of total antibiotic (Tompsett *et al.*, 1947; Kunin, 1961; Colville & Quinn, 1961; Bond *et al.*, 1963). Therefore, in the evaluation of an antibiotic, due consideration should be given to the extent of binding to serum proteins, as well as to data relating to *in vitro* and *in vivo* activity and to studies on absorption and excretion.

SUMMARY

1. The binding of penicillins and other antibiotics in serum was studied by ultrafiltration techniques.

2. The nature of the binding was shown to be essentially reversible. The extent of binding was largely independent of the concentration of antibiotic below 100 μg/ml., but above this concentration binding decreased with increase in drug concentration. Binding of penicillins was little influenced by temperature in the range 4 to 37° C.

3. Results were obtained on the relationship between the extent of binding and the concentration of serum. The extent of binding of benzylpenicillin and cloxacillin was also determined in the serum of different animal species. With both compounds, binding was greatest in human serum. No significant variation was found in the extent of serum binding between different human subjects.

4. Competitive binding was demonstrated between cloxacillin and other compounds bound to serum proteins including phenylbutazone, sodium salicylate and sulphonamides.

5. Diminution of antibacterial activity of penicillins in the presence of serum was directly proportional to the extent of binding as determined by ultrafiltration.

6. Results are presented for the extent of binding in normal human serum of various antibiotics including all the penicillins at present in clinical use.

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