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THE BINDING OF PENICILLINS TO BOVINE SERUM ALBUMIN

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Abstract—The binding of phenoxymethylpenicillin and benzylpenicillin to bovine serum albumin has been studied over the pH range 1·5–10·0 in a variety of buffers. There was a marked reduction in binding above pH 9. The buffers used interfered with binding in the order trismaleate > veronal = chloride > phosphate. Buffer interference caused an anomalous reduction of binding between pH 9 and pH 6·5.

At pH 7·4 phenoxymethylpenicillin was bound to bovine serum albumin at one main site and numerous subsidiary ones. The main site carried a little over half of the bound penicillin. These sites are discussed with reference to the binding sites which have been found for other anionic substances.

Continuous dialysis indicated that the binding was completely reversible.

The possible nature of the penicillin-binding sites on albumin is discussed in the light of these findings.

MANY therapeutically-useful drugs are bound to plasma proteins and this binding may profoundly affect their properties.[1, 2]

In 1950 Klotz and his co-workers[3] studied the binding of serum albumin to the penicillins which were available at that time. They found that the penicillin/albumin bond was a reversible one, that in a series of penicillins binding increased with increase of molecular weight and that the binding of benzylpenicillin was maximal at pH 6·2. No study was made of the number of binding sites on the albumin molecule or of their nature. Many of the more recently-developed penicillins also show a high degree of binding in plasma which affects their antibacterial activity *in vitro*[4] and *in vivo*.[5] In the work reported here an attempt has been made to elucidate the nature of the penicillin-binding sites on serum albumin as a preliminary to studying the ways in which penicillin may be displaced from these sites.

EXPERIMENTAL

Methods

1. *Equilibrium dialysis.* Dialysis was carried out in sacs prepared from Visking tubing ($\frac{8}{32}$ in.) that had been soaked overnight in buffer. Superficial water was squeezed from the membrane before use. One end of the membrane was closed with a double knot. Albumin solution (6 ml) in buffer was introduced into the sac and the other end was tied off. The outside of the sac was washed by dipping momentarily into buffer and the sac was then placed in a test-tube containing 11 ml of buffer to which penicillin had been added. The test-tube was closed with a silicone-rubber bung. The tube and its contents were then agitated by rocking at 4° for 18 hr. At the end of this period the

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diffusate was tested to ensure that no albumin had leaked from the bag. The concentration of penicillin was then determined in both the albumin solution and the diffusate. Thus no correction for penicillin-binding by the Visking sac was necessary.

The adequacy of the 18-hr period for the attainment of equilibrium was shown as follows. Two dialysis tubes were made up: in one the penicillin was placed in the albumin solution and in the other the penicillin was added to the buffer. After 18 hr dialysis the concentration of penicillin in the albumin solution was the same in each tube, showing that equilibrium had been attained.

2. *Penicillin 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 $\pm 5\%$ at $p = 0{\cdot}95$. When standard penicillin solutions containing a constant concentration of albumin were assayed zone diameters were reduced but the dose/response relationship was still linear. Therefore albumin was added to all standards and to samples where necessary so that the albumin concentration in each solution applied to the plate was the same.

3. *Estimation of sodium and chloride.* Sodium concentrations were measured in an EEL flame photometer and chloride concentrations by the method of van Slyke.[6]

4. *Estimation of albumin.* Albumin was estimated by the biuret method.[7] An SP 500 spectrophotometer was used to measure absorption at $\lambda = 540$ mμ.

5. *Measurement of pH.* pH was determined in an EIL 23 A pH meter using a miniature glass electrode.

6. *Calculation of binding in dialysis experiments.* The concentration of penicillin in each solution was obtained by applying a correction factor to take into account the space occupied by the protein and other solutes. Thus [penicillin$_{albumin}$] was multiplied by the correction factor

$$f = \frac{100}{[99{\cdot}6 - 0{\cdot}75\ (\text{g albumin}/100\ \text{ml})]},$$

this assumes that the specific gravity of albumin is 1·33 and that 0·4% of the volume of the solution is occupied by solutes other than protein.[8] Similarly [penicillin$_{diffusate}$] was multiplied by $\frac{100}{99{\cdot}6} = 1{\cdot}004$ to allow for the space occupied by the buffer solutes.

Because of the Donnan effect [penicillin$_{diffusate}$] over-estimates the concentration of free penicillin in the albumin solution. The Donnan correction factor r, was calculated from the distribution of sodium between the albumin solution and the diffusate. Thus

$$r = \frac{[Na^+_{albumin}]}{[Na^+_{diffusate}]}$$

(the use of r is discussed more fully in the Results section.). As the magnitude of r will vary with protein concentration, molarity and pH, it was estimated individually for each tube and the value obtained was used in the calculations for that tube. The extent of binding was calculated as follows:

$$[\text{penicillin}_{bound}] = [\text{penicillin}_{total}] - [\text{penicillin}_{free}]$$
$$= [\text{penicillin}_{albumin}] \times f - \frac{[\text{penicillin}_{diffusate}] \times 1{\cdot}004}{r}$$

Materials

1. *Penicillins.* Benzylpenicillin = Penicillin G (Glaxo Laboratories Ltd.) and phenoxymethylpenicillin = Penicillin V [Distillers Co. (Biochemicals) Ltd.] were used.

2. *Albumin.* Bovine serum albumin (Fraction V) supplied by the Armour Pharmaceutical Co. was used.

3. *Buffers.* 0·15 M buffers were made by mixing the appropriate volumes of the following solutions:

(a) *phosphate*—0·15 M solutions of NaH_2PO_4 and Na_2HPO_4;

(b) *bicarbonate*—0·15 M solutions of Na_2CO_3 and $NaHCO_3$;

(c) *veronal*—10 ml 0·15 M sodium acetate + 10 ml 0·15 M barbitone sodium + required amount of 0·15 N HCl + 0·15 M NaCl to 100 ml and

(*d*) *tris-maleate*—Equal volumes of 0·15 M tris(hydroxy methyl) aminomethane and 0·15 M maleic acid mixed and required amount of 0·15 N NaOH added.

0·03 M buffers are made in an analogous manner.

4. *Sodium chloride solution.* The total chloride concentration was kept constant as follows:

(i) at pH's above 4·8 (the iso-electric point of the albumin) the appropriate amount of 0·15 N NaOH was diluted to 50 ml and 50 ml 0·3 M NaCl was added.

(ii) at pH's below 4·8 the appropriate amount of 0·15 N HCl was made up to 100 ml with 0·15 M NaCl.

RESULTS

1. *The Donnan Effect*

Phenoxymethylpenicillin and benzylpenicillin are acids with pK_a's of 2·7[9] and the distribution of ionised penicillin across a Visking membrane in the presence of protein will be modified by the Donnan effect. Thus some measure of the Donnan ratio is required.

The Donnan ratio was calculated (r_{calc}) from Tanford's[16] data on the net charge on the albumin molecule at different pH's. It was also estimated from the distribution of sodium (r_{Na}) and chloride (r_{Cl}) in experiments in which 0·15 M NaCl was dialysed in the presence of $6{\cdot}86 \times 10^{-4}$ M albumin over the pH range 1·5 to 10.

$$r_{Na} \text{ and } r_{Cl} \text{ are given by } \frac{[Na^+_{albumin}]}{[Na^+_{diffusate}]} \quad \text{and} \quad \frac{[Cl^-_{diffusate}]}{[Cl^-_{albumin}]}$$

respectively. The results are shown in Fig. 1.

The deviations of r_{Na} and r_{Cl} from r_{calc} can be attributed to binding of chloride by albumin.[10] Binding of chloride will lower r_{Cl} by increasing $[Cl^-_{albumin}]$ and will raise r_{Na} by increasing the net charge on the albumin molecule. Thus, providing sodium itself is not bound, r_{Na} is a true measure of the Donnan effect under these conditions. Now the deviations of r_{Na} and r_{Cl} from r_{calc} could also be attributed to binding of sodium. Unfortunately one cannot determine whether the unequal distribution of sodium across a Visking membrane is partly due to binding or whether it is due solely to the Donnan effect. However Scatchard,[11] using a potentiometric method, showed that sodium is not bound to albumin at pH 7·4 and it is reasonable to assume that it will not be bound at pH's below 7·4 at which the albumin becomes increasingly electropositive. Thus in the penicillin-binding experiments r_{Na} has been used as a

measure of the Donnan effect (see Experimental section for details of the calculations). There could admittedly be a small error at pH's above 7·4 where some binding of sodium *could* occur although there is no evidence that it does. It will be seen from Fig. 1 that r_{Na} is unity at pH 4·75, suggesting that this is the isoelectric point of the albumin under these conditions.

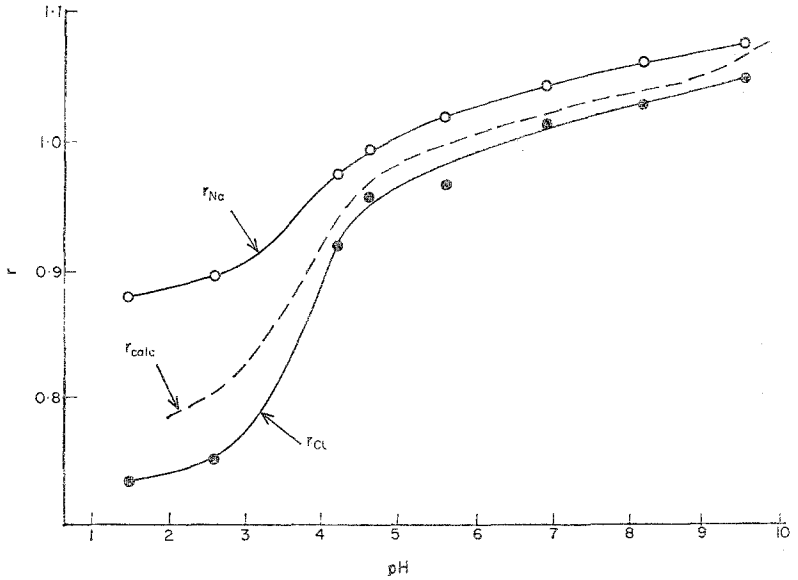

FIG. 1. The distribution of sodium and chloride across a Visking membrane when 0·15 M NaCl was dialysed in the presence of 6·86 × 10^{-4} M albumin over the pH range 1·5–10·0

$$\bigcirc\ r_{Na} = \frac{[Na^+_{albumin}]}{[Na^+_{diffusate}]}; \qquad \bullet\ r_{Cl} = \frac{[Cl^-_{diffusate}]}{[Cl^-_{albumin}]}$$

The broken line represents the Donnan ratio which would be expected if neither ion were bound.

2. *The effects of change of pH and buffer composition on binding*

The binding of 6·42 × 10^{-5} M phenoxymethylpenicillin and benzylpenicillin to 6·86 × 10^{-4} M albumin was studied over the pH range 1·5 to 10·0 by dialysis in 0·15 M sodium chloride (pH 1·5–4·3), 0·15 M veronal buffer (pH 4·8–8·5) and 0·15 M bicarbonate buffer (pH 8·2–10). The results are shown in Fig. 2. Bound penicillin is expressed as a percentage of the total present in the albumin solution.

Phenoxymethylpenicillin has a pK_a of 2·7 and hence at pH's below 5 will no longer be completely ionised. The broken line in Fig. 2 shows the binding expressed as a percentage of the total ionised penicillin present, i.e.

$$\frac{100 \times [\text{penicillin}_{\text{bound}}]}{[\text{penicillin}_{\text{bound}}] + [\text{ionised penicillin}_{\text{free}}]}$$

This suggests that the carboxyl group of the penicillin may have to be dissociated for binding to take place and that the reduction in binding at low pH's may be due to a reduction in the concentration of ionised penicillin. The reversible expansion of albumin which occurs below pH 4·3[12] could also contribute to changes in binding at low pH.

A notable feature of the curves in Fig. 2 is the dip that occurs between pH 4 and pH 9. Phenoxymethylpenicillin-binding was studied further over this mid-pH range in 0·15 M phosphate and tris-maleate buffers and in 0·15 M sodium chloride. The results are shown in Fig. 3 together with the results previously obtained using 0·15 M veronal buffer.

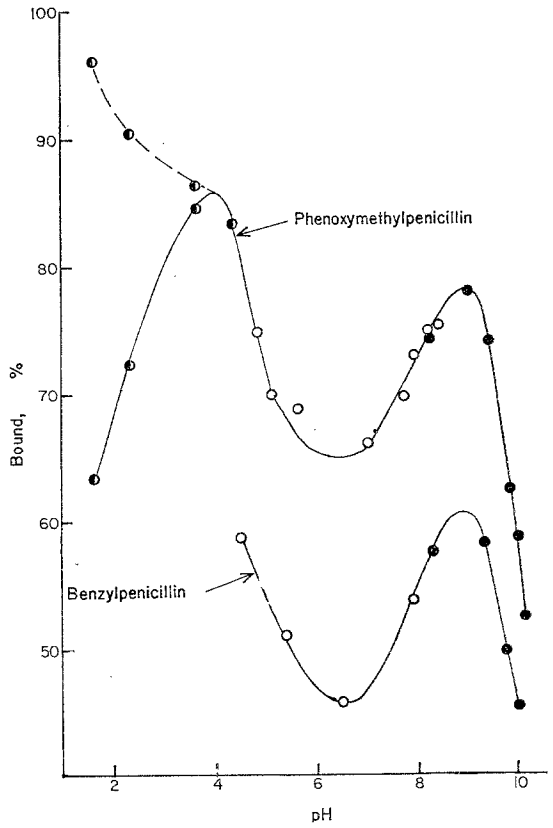

FIG. 2. The effect of pH on the bindingof phenoxymethylpenicillin and benzylpenicillin to albumin.
◐ = in 0·15 M NaCl; ○ = in 0·15 M veronal buffer;
● = in 0·15 M bicarbonate buffer;
— — — — = binding as a percentage of total ionised phenoxymethylpenicillin. Note suppression of zero on ordinate.

These buffers interfere with binding in the order tris-maleate > veronal = chloride > phosphate. In so far as they overlap these results show the same pattern as previous studies which found that buffers interfered with methyl orange-albumin binding in the order veronal > chloride > phosphate.[13] Phenol red also was more highly bound to albumin in phosphate than in veronal buffer.[14]

The effect of electrolyte concentration was determined by measuring the binding of phenoxymethylpenicillin in 0·03 M NaCl (Fig. 4).

This reduction of electrolyte concentration increases the magnitude of the Donnan effect. However this should not affect the results because, as noted in the Methods

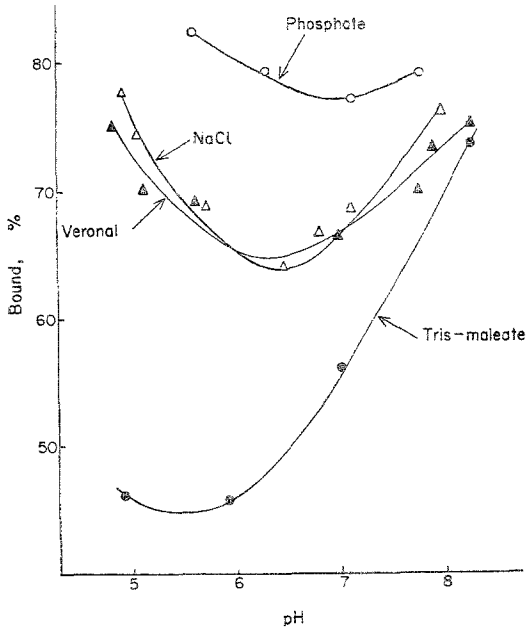

FIG. 3. The effect of pH on the binding of phenoxymethylpenicillin to albumin.
○ = in 0.15 M phosphate buffer; △ = in 0·15 M NaCl;
▲ = in 0·15 M veronal buffer; ● = in 0·15 M trismaleate buffer.
Note suppression of zero on ordinate.

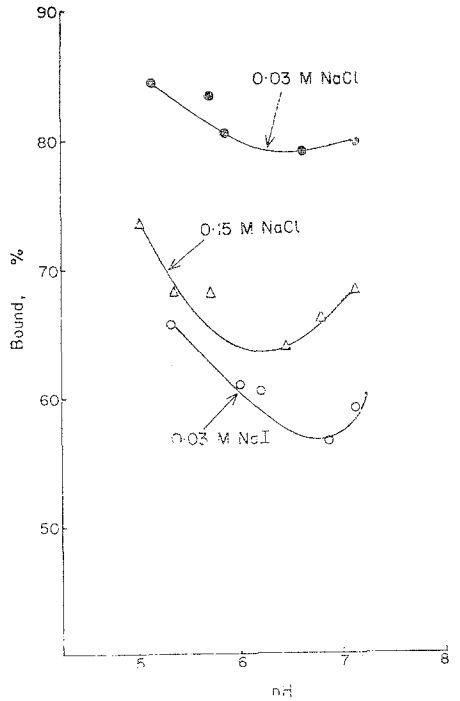

FIG. 4. The effect of pH on the binding of phenoxymethylpenicillin to albumin.
● = in 0·03 M NaCl; ○ = in 0·03 M NaI;
△ = in 0·15 M NaCl.
Note suppression of zero on ordinate.

section, the Donnan correction was determined individually for each tube. The observation that 0·03 M NaCl interfered with binding to a lesser extent than 0·15 M NaCl could be attributed to the reduction in [Cl^-] or to some other effect of reduced molarity as such. However 0·03 M NaI interfered with binding to a greater extent than 0·15 M NaCl (Fig. 4). This suggests that the extent of the interference depends both on the nature of the competing anion and on its concentration. Iodide is more highly bound than chloride[10] and so might be expected to produce a greater degree of interference.

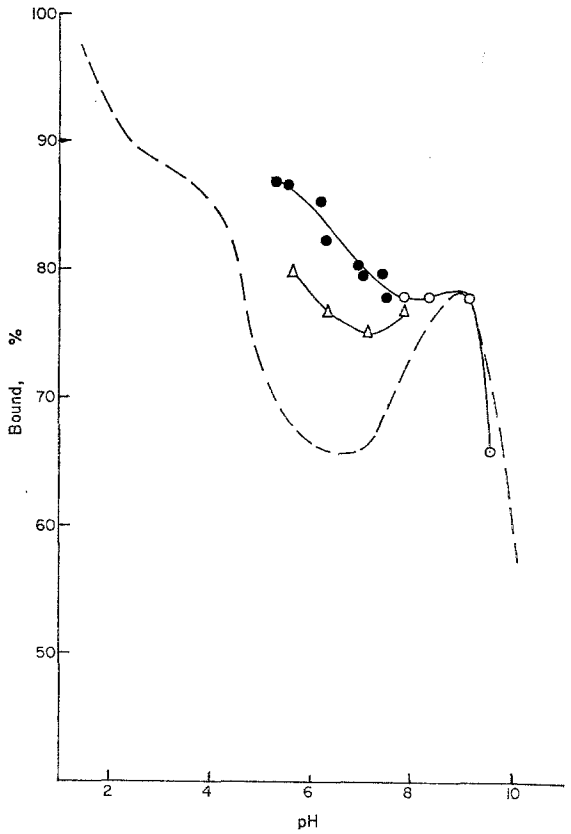

FIG. 5. The effect of pH on the binding of phenoxymethylpenicillin to albumin.
● = in 0·03 M phosphate buffer;
△ = in 0·15 M phosphate buffer;
○ = in 0·03 M bicarbonate buffer;
— — — — taken from Fig. 2.
Note suppression of zero on ordinate.

Figure 5 shows the binding of phenoxymethylpenicillin in 0·15 M and 0·03 M phosphate buffers and in 0·03 M bicarbonate buffer. The continuous line represents the binding in 0·15 M buffers taken from Fig. 2. Phosphate buffer interfered less with binding than the other buffers used here. The form of the curves obtained in 0·03 M phosphate and bicarbonate buffers is probably the most reliable.

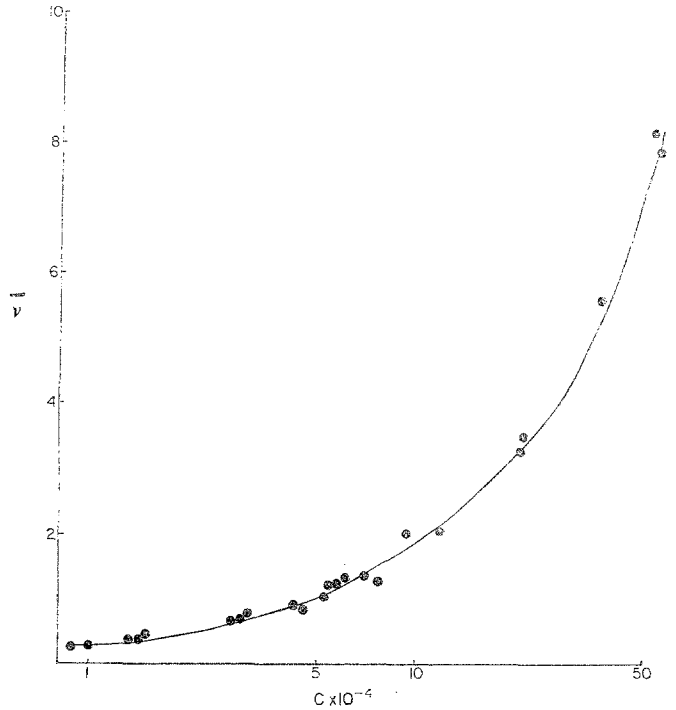

FIG. 6. The binding of phenoxymethylpenicillin to albumin in 0·08 M phosphate buffer, pH 7·4.
c = concentration of free phenoxymethylpenicillin;
$\bar{\nu}$ = moles phenoxymethylpenicillin bound per mole of albumin.

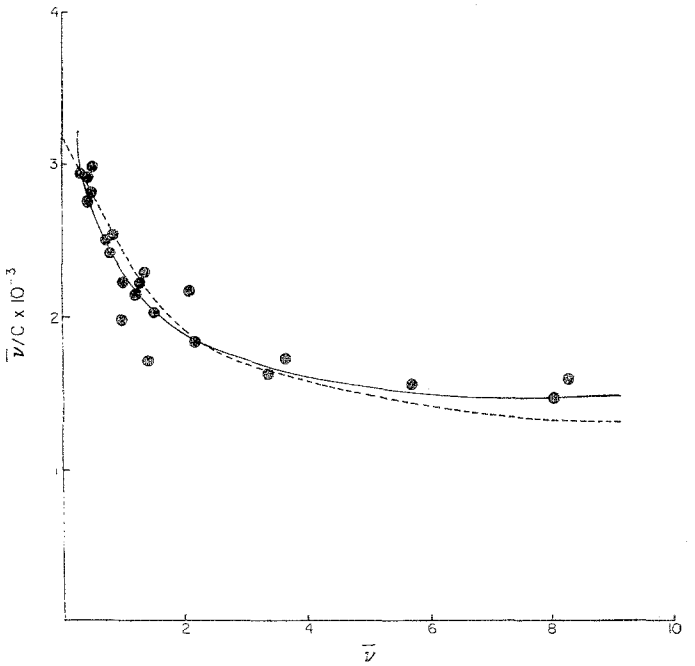

FIG. 7. The binding of phenoxymethylpenicillin to albumin in 0·08 M phosphate buffer, pH 7·4
— — — — corresponds to $n_1 = 1$, $k_1 = 1887$, $n_2 = 3$, $k_2 = 14{\cdot}0$, $n_3 = 82$, $k_3 = 15{\cdot}2$;
———— corresponds to $n_1 = 0{\cdot}78$, $k_1 = 2376$, $n_2 = 86{\cdot}8$, $k_2 = 16{\cdot}2$.

The decreased binding which occurs in the presence of 0·15 M buffers between pH 4 and pH 9 is apparently the result of competition from the buffer anion, for it is most pronounced in the presence of those buffers which interfere most markedly with penicillin-binding and appears to be minimal in the case of 0·03 M phosphate buffer.

3. *The binding of phenoxymethylpenicillin to albumin at pH* 7·4

The binding of phenoxymethylpenicillin to albumin at pH 7·4 was determined by equilibrium dialysis in 0·08 M phosphate buffer. The concentration of albumin was varied between $1{\cdot}18 \times 10^{-4}$ M and $7{\cdot}54 \times 10^{-4}$ M and the amount of phenoxymethylpenicillin added was such that the final concentration of free penicillin ranged from $8{\cdot}4 \times 10^{-5}$ M to $5{\cdot}4 \times 10^{-3}$ M. In Fig. 6 the concentration of free penicillin (c) is plotted on a log scale against the moles of penicillin bound per mole of albumin ($\bar{\nu}$), assuming a molecular weight of 65,000 for albumin.

The same relationship between $\bar{\nu}$ and c holds for all concentrations of albumin. In the case of methyl orange-albumin binding, however, this relationship varied with protein concentration.[13]

Scatchard[15] has pointed out that the relationship between the concentrations of free and bound penicillin can be expressed in the form:

$$\bar{\nu}/c = kn - k\bar{\nu} \tag{1}$$

where n is the number of binding sites per albumin molecule and k is the association constant. If the sites are homogeneous a graph of $\bar{\nu}/c$ plotted against $\bar{\nu}$ should be a straight line with intercepts nk and n. When the data are plotted in this way the points fall on a curve (Fig. 7).

This deviation from ideal behaviour could be attributed to repulsion of oncoming penicillin ions by those already bound. The corrected form of equation (1) applicable in this situation is as follows:[15]

$$\bar{\nu}/c \exp 2w(z_p + \bar{\nu}) = kn - k\bar{\nu} \tag{2}$$

where z_p is the net charge on the protein, taken as 12,[16] and w is the Debye-Huckel parameter, taken as 0·026.[16] However, equation (2) does not fit the observations either since, as shown in Fig. 8, the graph of $\bar{\nu}/c \exp 2w(z_p + \bar{\nu})$ plotted against $\bar{\nu}$ is not linear and in fact the slope is reversed at higher concentrations.

This would only occur if an ion, once bound, facilitated the binding of successive ions. The form of Fig. 8 resembles that obtained in similar circumstances by Scatchard with data for the binding of azosulphathiazole to albumin.[15] He suggested that the electrostatic correction might be inapplicable because the anion was displacing buffer anion from the binding sites rather than reacting with uncombined albumin. In fact the necessary correction may be in the opposite direction because if the penicillin displaces a HPO_4^{2-} ion the change in net charge will be $+1$.[17] It must be concluded, then, that the curve obtained when the data are plotted according to equation (1) (Fig. 7) is due to the existence of a number of sites each with a different affinity for the penicillin.

Scatchard[15] used a graphical method for finding the association constants for a number of heterogeneous binding sites. By a series of successive approximations he

found the values of k which gave the best fit of the equation

$$\frac{\bar{\nu}}{c} = \sum \frac{k}{1 + kc} \tag{3}$$

to his plot of $\bar{\nu}/c$ vs. $\bar{\nu}$. However in the work reported here eqn. (3) was solved on a computer by the method of least squares, which has the advantage of being less laborious and more precise than the graphical method. The computer was programmed

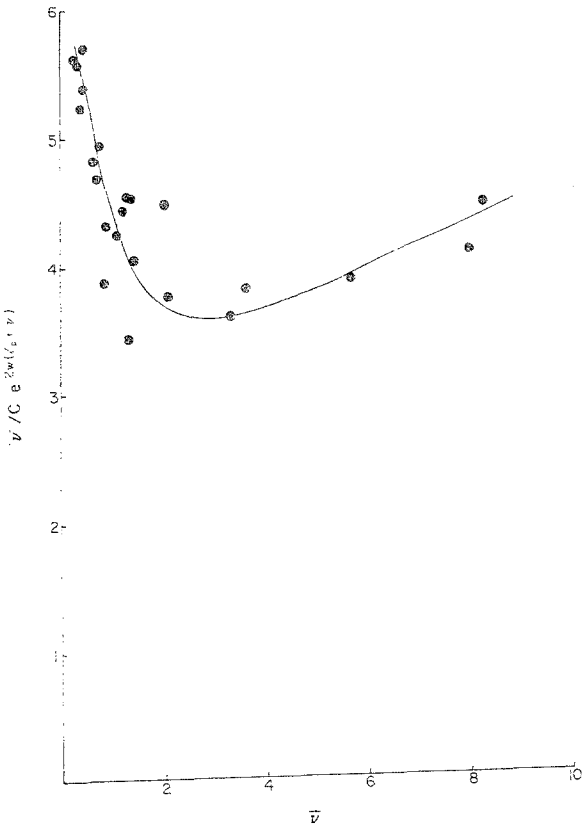

FIG. 8. The binding of phenoxymethylpenicillin to albumin in 0·08 M phosphate buffer, pH 7·4, plotted on the assumption that there was electrostatic interaction between bound molecules.

to solve the expression for a finite number of terms. By extrapolation the total number of sites appeared to be of the order of 90 which justified the assumption that, as an initial approximation, the total number of sites was 86, which is the number of basic groups on the albumin molecule at pH 7·4.[16] The following equation was solved for the data:

$$\frac{\bar{\nu}}{c} = \left(\sum_{j=1}^{4} \frac{k_j}{1 + k_j c} \right) + \frac{82\, k_5}{1 + k_5 c} \tag{4}$$

The values obtained were: $k_1 = 1887$; $k_2 = k_3 = k_4 = 14{\cdot}0$ and $k_5 = 15{\cdot}2$. Substitution of these values in eqn. (4) gives the broken line in Fig. 7. There would appear to be a single binding site with a high affinity for penicillin and a large number of other sites which cannot be distinguished from each other from the available data. Assuming that there are only two distinguishable groups of sites the binding relationship may be expressed in the form

$$\frac{\bar{v}}{c} = \frac{n_1 k_1}{1 + k_1 c} + \frac{n_2 k_2}{1 + k_2 c} \tag{5}$$

as suggested by Karush.[19] If eqn. (5) is solved by computer the binding sites may be estimated without resorting to extrapolation. The values obtained for n_1 and n_2, however, will not be integers. The best values obtained by solving this equation for the data are shown in Table 1.

TABLE 1. VALUES FOUND FOR THE NUMBER OF BINDING SITES (n) ON THE ALBUMIN MOLECULE TOGETHER WITH THEIR ASSOCIATION CONSTANTS (K) FOR PHENOXYMETHYLPENICILLIN

	n	K
Primary binding site	0·78	2376
Subsidiary sites	86·8	16·2

These values are plotted as a continuous line in Fig. 7, and are a significantly better fit, as judged by the sum of squares of the deviations, than those obtained in the calculations that assumed that n_1 was an integer.

It seems then that bovine albumin carries one site with a high affinity for penicillin and a large number of subisidiary sites of a very low affinity. The fact that n_2 corresponds closely with the total number of basic groups present at pH 7·4 (Table 3) is probably fortuitous as the extrapolation is so large as to be meaningless.

Assuming that ox plasma contains 3·63 per cent albumin[19] the values for n and K in Table 1 predict that, at plasma concentrations obtained *in vivo*, 64 per cent of the penicillin would be bound. This agrees well with the extent of binding in bovine plasma as measured by ultrafiltration[20] and confirms observations[21] that binding to other proteins in plasma is negligible.

4. *Continuous dialysis*

A $2{\cdot}57 \times 10^{-3}$ M solution of phenoxymethylpenicillin in $6{\cdot}86 \times 10^{-4}$ M albumin was dialysed against running tap water. The penicillin remaining in the dialysis sac after various periods of time was assayed against a standard which had also been kept at the temperature of tap water. To allow for dilution of the sac contents caused by influx of water the penicillin concentration was related to albumin concentration. The results are shown in Fig. 9. The concentration of penicillin fell exponentially indicating that the binding to albumin was fully reversible.

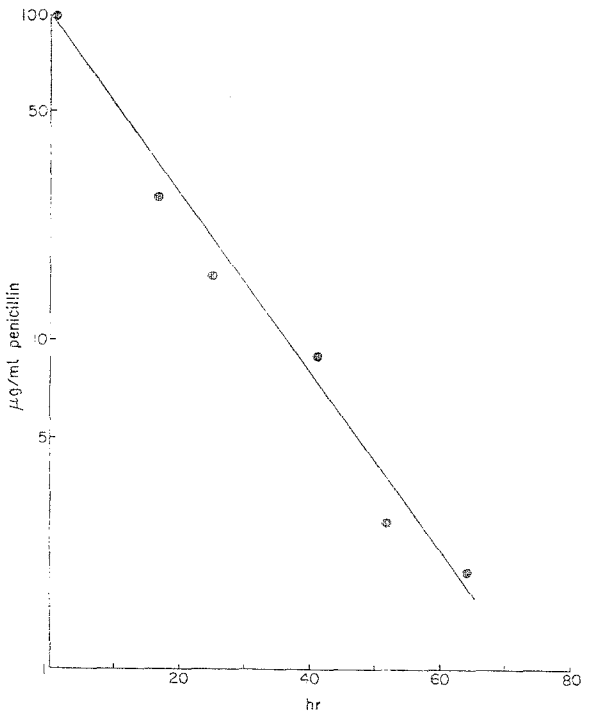

FIG. 9. The continuous dialysis of phenoxymethylpenicillin against tapwater.

DISCUSSION

1. *Buffer effects*

As the pH of a protein solution is lowered more basic groups become available on the protein to bind an anion and at the same time the number of acidic groups, tending to repel the anion, is reduced. Thus one would expect a general tendency for anion-binding to increase at lower pH values. However an anomalous reduction of binding with lowering of pH has been reported for a number of anions. The binding of four sulphonamides in plasma fell over the pH range 9–5[22] and five barbiturates showed maximal binding to albumin at pH 8 with less binding at lower and higher pH values.[23] The barbiturates and sulphonamides are weak acids and so the reduced binding at lower pH's could have been due to reduction in the amount of drug present in the ionised form. However methyl orange was more extensively bound to albumin at pH 9·2 than at pH 6·8[24] and benzyl-penicillin was more highly bound at pH 6·2 than at pH 5[3] and, unlike the barbiturates and the sulphonamides, these are strong acids which would have been fully ionised over the pH range in question. Klotz[24] attributed this anomalous effect of pH to a change in the tertiary structure of the protein around pH 7. The results reported here suggest an alternative explanation, namely that it may be due to buffer competition. However it should be noted that, although buffer competition seems to explain the anomalous reversal in the present studies, Karush[25] found a small reversal in the binding of methyl orange between pH 7·6 and pH 5·3 although there was no buffer present in the system used.

2. *Number and nature of binding sites*

Continuous dialysis (Fig. 9) shows that the penicillin-protein bond is fully reversible. The penicillin is apparently bound by means of its ionised carboxyl group so that at pH's below 5, when this is no longer fully dissociated, binding decreases. The reduction of penicillin-binding which occurs above pH 9 suggests that binding is to basic groups on the protein. Further evidence that binding is to basic sites is the finding that other highly-bound anionic drugs displace penicillin whereas highly-bound cationic drugs do not.*

Phosphate (0·03 M) and bicarbonate buffers produce least interference with penicillin-binding and so the form of the curve obtained with these buffers is probably the most reliable (Fig. 5).

The sharp decrease in binding at pH 9 is unlikely to be due to a gross change in the structure of the albumin molecule, which is stable up to a pH of 10·5,[12] and more probably represents a change in the penicillin-binding site itself. Calculations of the number of ionised groups on the albumin molecule at different pH's from the results of Tanford[16] suggest that the fall in binding at pH 9 might be caused either (i) by the closure of ϵ-amino groups to which the penicillin was bound or (ii) by the ionisation of a phenolic hydroxyl group adjacent to the binding site. Figure 5 also shows that, as hydrogen ion concentration is increased below pH 5, binding continues to increase although no further ionisation of basic groups on the protein is taking place. This increase of binding in the low pH range may be due to closure of carboxyl groups which at higher pH's had tended to interfere with binding.

It may be informative to compare the values for n and k found here (Table 1) with those found in similar studies on the binding of other anionic substances. Many of these, unfortunately, have been carried out in the region of pH 5 (the isoelectric region of albumin) and so are not comparable with the present studies. Table 2 shows the estimates obtained in various studies carried out in the region of pH 7·4.

TABLE 2. VALUES FOUND BY VARIOUS AUTHORS FOR THE NUMBER OF BINDING SITES (n) ON THE ALBUMIN MOLECULE AND THEIR ASSOCIATION CONSTANTS (k) FOR DIFFERENT ANIONIC SUBSTANCES

Substance	pH	Albumin	Method	n_1	$k_1 \times 10^4$	n_2	$k_2 \times 10^4$	Reference
Caprylate	7·6	Bovine	Karush	5·0	1·22	31	0·014	27
Thiopentone	7·4	Bovine	Karush	1·25	6·0	20·75	0·06	23
Azo dye	7·0	Bovine	Karush	4·82	9·93	17·18	0·29	18
Methyl orange	7·45	Human	Scatchard	4	13·0	18	2·2	17
Phenylacetate	7·6	Bovine	Klotz	25	230			30
Oleate	7·45	Human	Scatchard	($n_0 = 2$, $K_0 = 11{,}000 \times 10^4$)				17
				5	400	20	0·1	

The form of the values obtained is governed by the method used for their calculation. The method of Klotz[26] will yield values for one set of binding sites only. Karush's method[18] makes the assumption that the binding sites fall into two, and only two, distinct groups and involves long extrapolations to obtain Σn and Σnz. It yields non-integral values for n. With the method of Scatchard[15] any number of groups of binding sites can theoretically be distinguished and integral values are obtained for n.

* Unpublished observation.

The values for thiopentone in Table 2 are probably unreliable because thiopentone has a pK_a of 7·6 and so will be less than half-ionised at pH 7·4. The other studies quoted in Table 2 agree in finding 4–5 primary sites and about 20 subsidiary ones. The figures given for phenylacetate binding are not inconsistent, having been calculated by a method that can distinguish only one set of sites. In the present study, on the other hand, we have found one primary site for penicillin and numerous subsidiary ones.

Subsidiary sites have also been found for chloride which at pH 5·3 was bound to 27 main sites and to about 70 other groups with smaller constants.[10] As with the present studies on penicillin the total number for chloride-binding sites approximated to the total number of basic groups ionised.

Goodman[17] found two high-affinity sites for oleate (designated n_0, K_0 in Table 2). However these sites had apparently been unmasked by pretreatment of the albumin to remove the two moles of fatty acid which are usually present. These high-affinity sites were not found in other studies of the binding of fatty acids using commercial albumin[27] and were specific for fatty acids in that they did not bind methyl orange.[17] Hence these sites are unlikely to be related to the single penicillin-binding site found in the present study using untreated commercial albumin. Also the values of K for the primary penicillin-binding site is of the same order as K for the 4–5 binding sites for other anions in Table 2 whereas K for the binding of oleate to its two specific sites was 10^4 times as great.

Thorp[2] has calculated that sulphinpyrazone, phenylbutazone and several sulphonamides may each, like penicillin, be bound at a single site on unrefined albumin. Unfortunately the calculations were made from measurements of binding in plasma and so necessarily involve uncertainties about the concentration of albumin, the magnitude of the Donnan effect and the occurrence of competing substances in plasma. In addition, only low concentrations of drug were used (in no case was $\bar{\nu} > 1$). This introduces further uncertainty about the validity of the values obtained for n and K because the form of the curve $\bar{\nu}/c$ vs. $\bar{\nu}$ is such that low drug concentrations give an accurate estimate of ΣnK (the intercept on the $\bar{\nu}/c$ axis) but underestimate Σn (the intercept on the $\bar{\nu}$ axis). Nevertheless Thorp's findings support the present study in that they indicate a single primary binding site for these substances. However there may well be additional subsidiary sites not detected at the drug concentrations used so that his estimate of K for the primary site may be too high. Single high-affinity groups in untreated albumin have also been found for two bipolar substances, thyroxine[28] and *l*-tryptophan.[29] The binding of the latter was stereospecific.

That the value obtained for n_1 in the present study is non-integral (0·78) is unlikely to be due to buffer competition because, although competition from buffer anions could lead to an underestimate of K, it should not affect the estimate of n. A fractional value of n would arise if the value of 65,000 used here for the molecular weight of albumin was too low; however a molecular weight of 83,000 would be needed to restore n_1 to unity. Karush[18] suggested that integral values for n cannot necessarily be expected as albumin may be heterogeneous. It is interesting to note that the α-amino group of the N-terminal aspartic acid residue is only partly ionised at pH 7·4 (Table 3) and so at this pH there would be a "statistical heterogeneity" of the albumin molecules, 80 per cent with an ionised α-amino group and the remainder without.

If the α-amino group were the primary penicillin-binding site this would account almost exactly for the value of 0·78 found for n_1. However, although this is an attractive explanation, calculations using the results in Table 1 show that 56 per cent of the bound penicillin is attached to the primary site and the remainder to the subsidiary sites. Thus if the α-amino group were the main site it would be carrying more than half of the bound penicillin and there would be a marked fall in the total bound over the pH range 7–9, when the group is closing. This does not occur (Fig. 5).

TABLE 3. THE IONISED GROUPS PRESENT ON THE ALBUMIN MOLECULE AT pH 7·4*

Residue	Group	Number per molecule	pK_a	Number ionised at pH 7·4
		Acidic groups		
C-terminal	α-carboxyl	1	3·75	1
Aspartic	β-carboxyl–	99	3·97	99
Glutamic	γ-carboxyl–			
Tyrosine	phenolic	19	10·35	0
			Total	100
		Basic groups		
Histidine	imidazole	16	6·9	6
N-terminal	α-amino	1	7·75	0·8
Lysine	ε-amino	57	9·8	57
Arginine	guanidine	22	>12	22
			Total	86

* Calculated from the data of Tanford.[16]

The fact that one site has a much greater affinity for penicillin than the others suggests that van der Waal's forces play a major part in binding. If they did, not all monovalent anions would presumably have similar affinities for all binding sites. The correlation between extent of binding and molecular weight for several series of homologous compounds[20, 23, 30] also suggests a role for van der Waal's forces. However these forces act only over very short distances so that initial ionic bonding is probably necessary in order to bring the non-polar parts of the drug molecule into sufficiently close apposition with auxiliary groupings on the albumin for van der Waal's bonds to be formed. Co-operation of ionic and van der Waal's forces has been shown in the case of *l*-tryptophan which is attached to albumin by ionic bonding through its carboxyl group and also by van der Waal's bonding of its indole ring.[29] The latter appears to contribute two-thirds of the bond energy.

Thus the identity of the primary penicillin-binding site on albumin is likely to reside largely in the nature of the groups adjacent to the basic group. The albumin molecule is a particularly flexible one and the binding of one mole of an anionic dye is known to alter its configuration.[31] It is possible that in the same way the binding of the first penicillin ion deforms the albumin molecule so as to bring auxiliary groups on the protein into an optimum spatial relationship for van der Waal's bonding. This could impose a rigidity on the albumin molecule which would preclude any similar rearrangement occurring for the binding of successive penicillin ions. These would

then be bound to other basic groups on the albumin molecule, with much less participation of van der Waal's forces and hence with much lower affinities. Thus a primary site would not pre-exist as such on the albumin molecule but would be partly a reflection of the ability of the drug, once it has been ionically bound, to hold parts of the albumin molecule in apposition to it. It is possible that more than one of the basic groups on the albumin is able to form the nucleus of the primary site. In this event there would be heterogeneity of penicillin-binding sites even if the albumin was homogeneous. In such a case mass-action expressions would be very difficult to interpret.

Simple molecules are likely to produce fewer van der Waal's bonds. They would thus produce less change in the configuration of the albumin and so might be equally well bound at a number of sites. For instance the fatty acids in Table 2 are likely to form van der Waal's bonds simply by lying with their carbon chain parallel to a carbon chain situated on an adjacent part of the albumin molecule.

The flexibility of albumin may help to explain how it can bind numerous very different anionic substances with high affinity but apparently very little specificity. High affinity is due to reinforcement by short-order van der Waal's forces and so requires that the site provides a close fit to the bound molecule. If the site is rigid this will imply that it will also be specific for this particular molecule. However if the site can adapt itself to a range of molecules it will bind each with high affinity and yet will show little specifiicity.

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