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THE DISPLACEMENT OF THREE ANIONIC DRUGS FROM BINDING TO BOVINE SERUM ALBUMIN BY VARIOUS ANIONIC COMPOUNDS

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Many drugs are bound to serum albumin and this binding profoundly affects their activity in the body (Goldstein, 1949; Thorp, 1964). The effect of protein-binding on the activity of antibacterial drugs is of especial interest. For instance, only free penicillin is able to diffuse from the blood into lymph (Verwey & Williams, 1962) and into milk (Rasmussen, 1959). In the same way protein-binding limits the passage of sulphonamides into cerebrospinal fluid (van Os, 1964). A number of substances displace penicillins (Kunin, 1965a) and sulphonamides (Anton, 1961) from plasma proteins *in vivo* and may thus be used to alter the distribution of these drugs in the body.

It is of interest, therefore, to study the way in which different drugs can be displaced from serum albumin and to determine whether antagonists displace all drugs with equal ease or whether they show specificity. In the work reported here the displacement of three unrelated anionic drugs, sulphamethoxypyridazine, phenoxymethylpenicillin and phenol red from bovine serum albumin has been investigated. For the measurement of protein-binding, ultrafiltration was preferred to equilibrium dialysis because ultrafiltration allows the required amounts of bound drug and antagonist to be added to the protein solution. In equilibrium dialysis, on the other hand, the drug added to the system is distributed between the buffer and protein compartments and hence the final concentration in the protein solution is uncertain.

METHODS

Albumin. Bovine Serum Albumin (Fraction V), supplied by the Armour Pharmaceutical Co., was used.

Drugs. Samples of the following preparations in powder form were obtained from the manufacturers: novobiocin ("Albamycin," Upjohn), phenoxymethylpenicillin ("Penicillin V," Abbott), phenylbutazone ("Butazolidin," Geigy), sulphinpyrazone ("Anturan," Geigy), probenecid ("Benemid," Merck Sharp & Dohme), sulphamethoxypyridazine ("Lederkyn," Lederle), tolbutamide ("Atrosin," Riker). The other substances used were commercially available. Acidic drugs were neutralized by addition of the appropriate volume of NaOH.

Buffer. Phosphate buffer was made by mixing 0.08 M solutions of Na_2HPO_4 and NaH_2Po_4 in the ratio 5.1:1 and adjusting the *p*H to 7.4 with NaOH or HCl.

Ultrafiltration. The apparatus used was similar to that described in a previous paper (Keen, 1965). Two modifications were introduced: (*a*) no provision was made for heating the apparatus

and all experiments were carried out at room temperature, and (*b*) ultrafiltration was effected by applying an air pressure of one atmosphere to the protein solution. This was less laborious than the negative-pressure method used previously.

Unless otherwise stated the bound drug was added to the albumin solution first, followed by the antagonist. The mixture was then allowed to stand for 30 min before it was ultrafiltered. This delay was probably unnecessary as Froese, Sehon & Eigen (1962) found that the time taken for a dye to be released from binding to protein was of the order of msec and Klotz, Triwush & Walker (1948) found that displacement of methyl orange by urethane was complete in ten min at the latest and possibly much earlier.

In a preliminary experiment a solution of each of the bound drugs in phosphate buffer was ultrafiltered. In no case did the concentration of the drug in the ultrafiltrate differ significantly from that in the original solution. This ensured that the membrane did not adsorb any of the drug or impede its passage relative to water.

Determination of drug concentrations. Each method of estimation was first tested to ensure that none of the antagonists used interfered with it.

(*a*) *Sulphamethoxypyridazine.* The method of Bratton & Marshall (1939) was used as a routine. However, phenylbutazone sulphinpyrazone and quinine interfered with the Bratton & Marshall reaction; samples containing these drugs were therefore assayed by the method of Morris (1941).

(*b*) *Phenoxymethylpenicillin.* Phenoxymethylpenicillin was assayed by the cup-plate method. Allowance was made for the effect of protein on the assay as previously described (Keen, 1965).

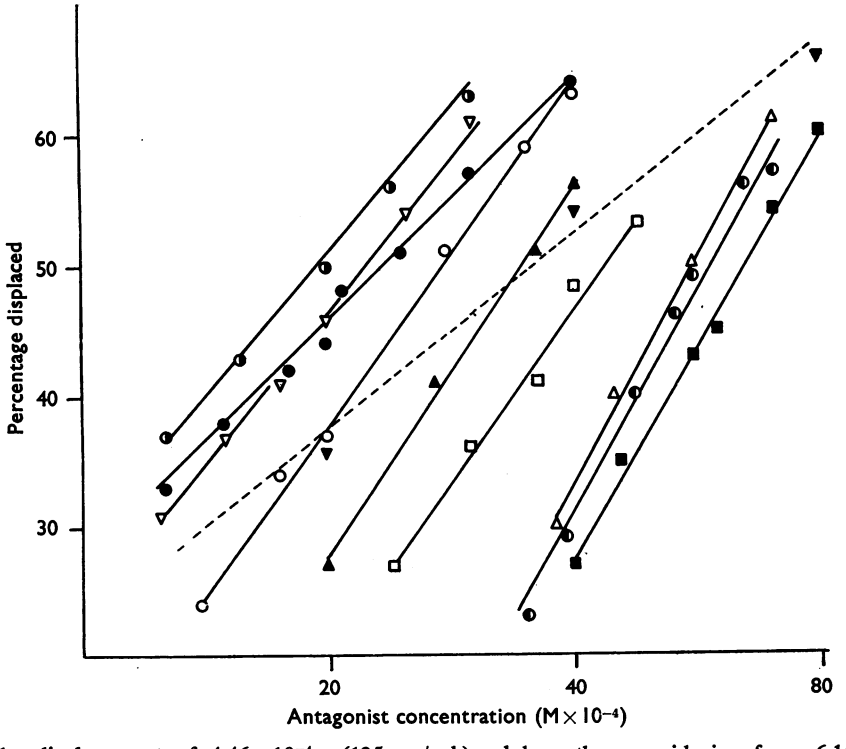

Fig. 1. The displacement of 4.46×10^{-4}M (125 μg/ml.) sulphamethoxy-pyridazine from 6.15×10^{-4}M (4%) bovine serum albumin by ten antagonists. △=caprylate; □=2:4 dichlorophenoxy-acetate; ▲=2:6 dihydroxybenzoate; ○=*p*-iodobenzoate; ◐=novobiocin; ◑=phenyl-butazone; ■=probenecid; ▼=salicylate; ●=sulphinpyrazone; ▲=tolbutamide;

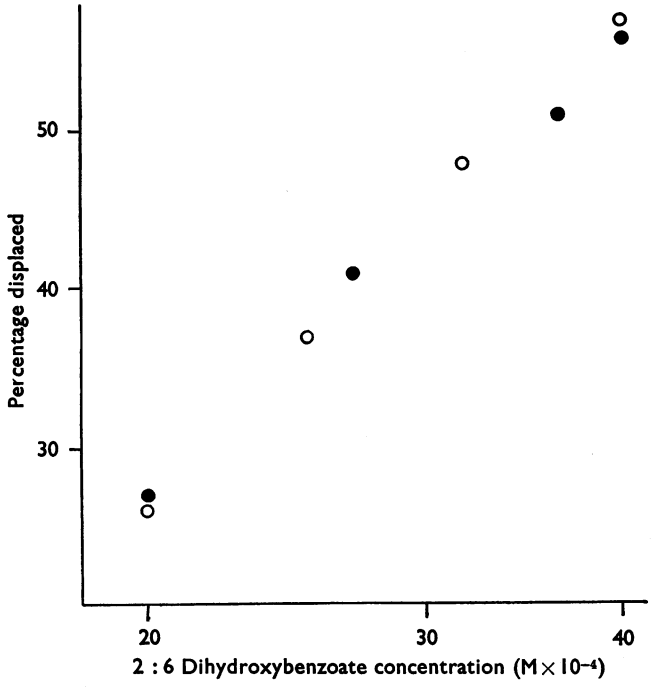

Fig. 2. The displacement of two concentrations of sulphamethoxypyridazine from 6.15×10^{-4}M (4%) bovine serum albumin by 2:6 dihydroxybenzoate. ○ = 4.46×10^{-4}M (125 μg/ml.) sulphamethoxypyridazine; ● = 1.25×10^{-4}M (35 μg/ml.) sulphamethoxypyridazine.

Novobiocin interfered with this microbiological assay; therefore, samples containing novobiocin were assayed by a modification of the micro-iodometric method of Novick (1962).

(c) *Phenol Red.* An SP 500 spectrophotometer was used to estimate phenol red in alkaline solution by its absorption at $\lambda = 555$ mμ.

Calculation of displacement. Percentage binding was calculated by the method previously described (Keen, 1965). This incorporates a correction for the Donnan effect and for the space occupied by the albumin. In measuring displacement a fixed concentration of the bound drug was used throughout while the concentration of antagonist was varied. The displacement produced by any particular concentration of antagonist was calculated as follows:

$$\frac{\%\text{ binding (antagonist absent)} - \%\text{ binding (antagonist present)}}{\%\text{ binding (antagonist absent)}} \times 100$$

Since the total concentration of bound drug remains constant this expression also measures the percentage of bound drug which has been displaced from the protein.

RESULTS

The ability of 10 anionic drugs to displace sulphamethoxypyridazine from binding to albumin was investigated. A series of solutions of 6.15×10^{-4}M (4%) albumin and 4.46×10^{-4}M (125 μg/ml.) sulphamethoxypyridazine in phosphate buffer were made up to contain a range of concentrations of each of the antagonists. The binding of sulphamethoxypyridazine in each solution was determined by ultrafiltration and the percentage displaced was calculated as described. The results are shown in Fig. 1. The

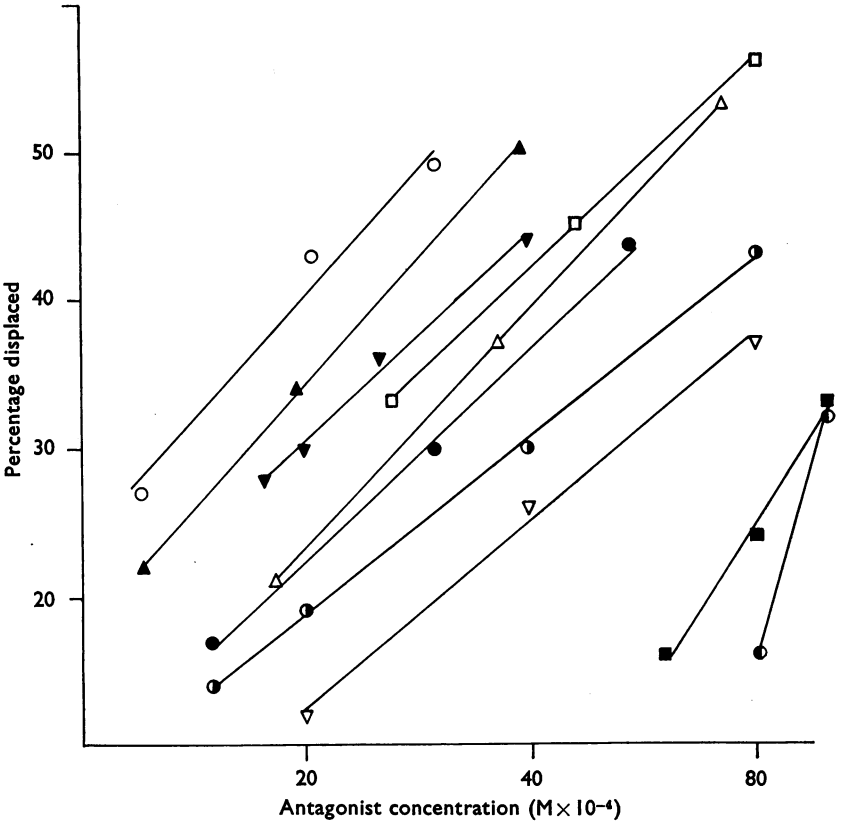

Fig. 3. The displacement of 3.22×10^{-4}M (125 μg/ml.) phenoxymethylpenicillin from 6.15×10^{-4}M (4%) bovine serum albumin. Symbols as in Fig. 1.

regression lines shown were calculated by the method of least squares. For nine of the agents these lines were substantially parallel. The response to salicylate was atypical and is indicated by a broken line in Fig. 1.

Three bases, quinine, tetraethylammonium and ephedrine were tested at a concentration of 80×10^{-4}M. None displaced sulphamethoxypyridazine.

The effect of varying the concentration of the bound drug was then tested. Fig. 2 shows the displacement of 4.46×10^{-4}M (125 μg/ml.) and 1.25×10^{-4}M (35 μg/ml.) sulphamethoxypyridazine by 2:6 dihydroxybenzoate. It will be seen that a given concentration of antagonist displaced the same percentage of bound sulphonamide at each sulphonamide concentration.

The displacement of phenoxymethylpenicillin and phenol red by the 10 anionic antagonists is shown in Figs. 3 and 4. In displacing phenol red and phenoxymethylpenicillin the mode of action of salicylate appeared to be similar to that of the other antagonists.

Since the regression lines in Figs. 2–4 are substantially parallel in each case they may be validly used to compare the activities of the different antagonists. Fig. 5 shows

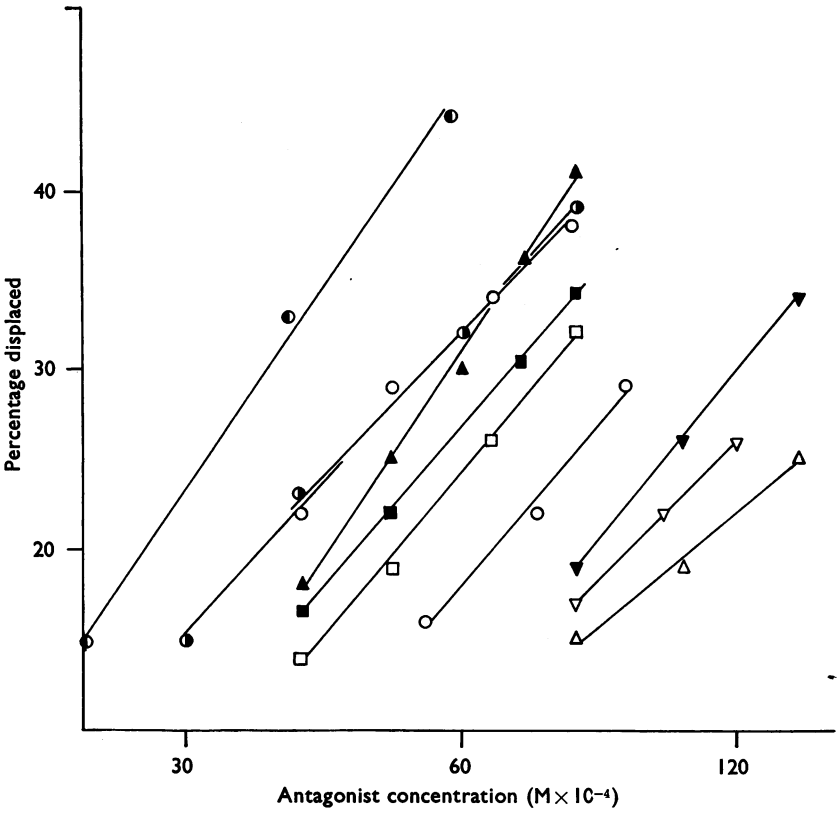

Fig. 4. The displacement of 3.54×10^{-4}M (125 μg/ml.) phenol red from 6.15×10^{-4}M (4%) bovine serum albumin. Symbols as in Fig. 1.

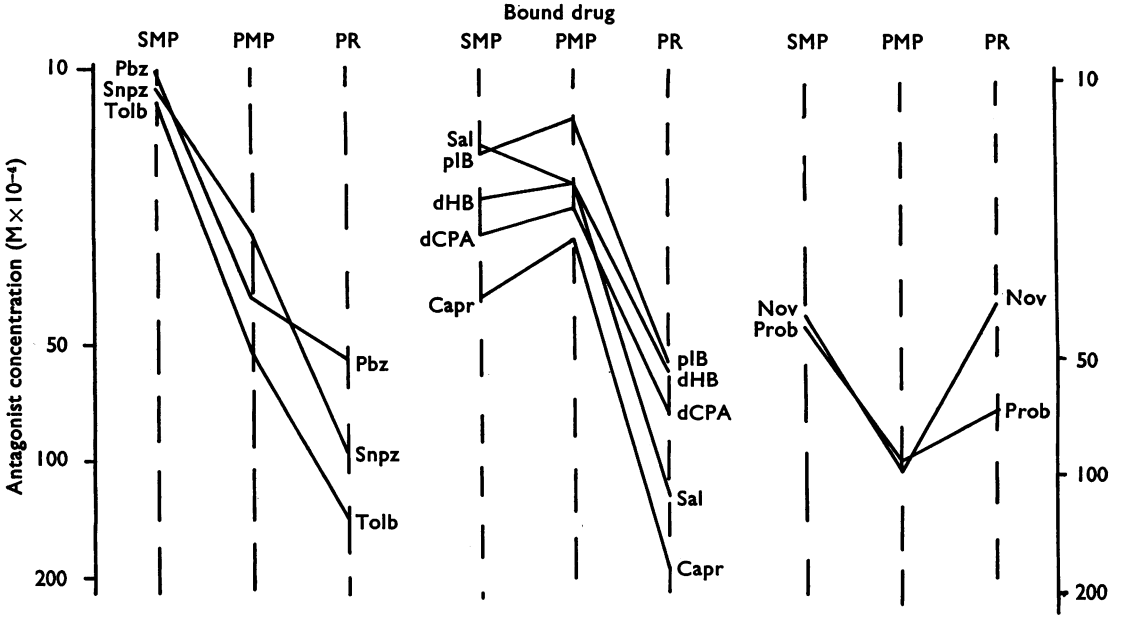

Fig. 5. Antagonist concentration giving 30% displacement. SMP = sulphamethoxypyridazine ; PMP = phenoxymethylpenicillin ; PR = phenol red ; Cap = caprylate ; DCPA = 2:4 dichlorophenoxyacetate ; DHB = 2:6 dihydroxybenzoate ; *p*IB = *p*-iodobenzoate ; Nov = novobiocin ; PBZ = phenylbutazone ; Prob = probenecid ; Sal = salicylate ; SNPZ = sulphinpyrazone ; Tolb = tolbutamide.

that concentration of each antagonist which displaces 30% of each bound drug, divided into three groups for greater clarity.

It will be seen that in general sulphamethoxypyridazine was more readily displaced than phenoxymethylpenicillin and that phenol red was less easily displaced than either, the mean antagonist concentrations being 23, 41 and 89×10^{-4}M respectively.

DISCUSSION

Sulphamethoxypyridazine, phenoxymethylpenicillin and phenol red are anionic and all of the 10 antagonists used are anionic drugs which have themselves been shown to be bound to serum albumin. On the other hand, three bases, quinine, tetraethylammonium and ephedrine did not displace sulphamethoxypyridazine. This confirms the finding of Anton (1961) that highly bound cationic drugs do not displace anionic ones and suggests that the drug/protein bond is primarily ionic in nature.

The antagonists used showed a degree of specificity. Phenoxymethylpenicillin was most readily displaced by simple molecules: benzoic acid derivatives and caprylate. Apparently these simple molecules were also able to gain access to the sites which bind sulphamethoxypyridazine and phenol red and thus showed little specificity. The best antagonists of sulphamethoxypyridazine were more complex molecules. Phenol red carries two acidic groups, only one of which is fully ionised at *p*H 7.4. It may be significant that the best antagonist of phenol red, novobiocin, also carries two acidic groups.

The specificity of displacement from serum albumin was of a very low order as compared with the specificity shown by drugs for effector receptors in tissues. For instance, the concentrations of *p*-iodobenzoate and probenecid which displaced 30% of phenoxymethylpenicillin from albumin differed by a factor of only 7 (Fig. 5) whereas the concentrations of mepyramine and atropine needed to block the histamine receptor in guinea-pig ileum differ by a factor of 10^4 (Schild, 1947). Nevertheless the specificity with which drugs are displaced from binding to albumin is important because the antagonist concentrations used are in the upper range of those attainable in plasma and thus only the most active antagonists are likely to reach high enough concentrations to displace a particular drug *in vivo*.

Antagonist specificity must imply that the three bound drugs are to some extent attached to different sites. The low order of specificity might be explained by postulating that the bound drugs and antagonists were each bound to a number of sites. The effectiveness of an antagonist would then depend on the extent to which its binding sites overlapped those of the drug it was displacing. However, the penicillin is probably attached to a single primary binding site (Keen, 1966). Thorp (1964) suggested that sulphamethoxypyridazine also is attached to a single main binding site. If this is so it is difficult to explain the gradation of displacing activity found here; for if sulphamethoxypyridazine and phenoxymethylpenicillin were attached to the same main site the antagonist which had the greatest affinity for this site should be the most active in displacing both drugs. Alternatively if the two drugs were attached to different binding sites there should be no overlap of displacing activity. However, this line of reasoning assumes that the sites to which the phenoxymethylpenicillin and

sulphamethoxypyridazine are bound are rigid sites pre-existing on the albumin molecule so that each antagonist will have a constant affinity for this site regardless of what other drugs are present. Now it has been suggested (Markus & Karush, 1958; Keen, 1966) that drugs to some extent form their own binding sites by distorting the albumin molecule to their own shape. If this were so the affinity of an antagonist for albumin would vary according to the drug with which it was competing because the antagonist's affinity would depend on its ability to distort the albumin molecule in the presence of the other drug. This would explain how a series of antagonists could show a low order of specificity in displacing two drugs each of which was bound to a single site.

Anton (1961) studied the ability of antagonists to displace another sulphonamide, sulphaethylthiadiazole, from albumin by adding similar amounts of each antagonist to a dialysis system and comparing the percentage displacement. This measure will differ from the one used here because the antagonist and the displaced sulphonamide can diffuse into the buffer compartment thus lowering the concentration of each in the protein solution. Hence the degree of displacement will vary according to the size of the buffer compartment. Anton (1961) used a single dose of antagonist and so there is no indication of behaviour at higher and lower antagonist concentrations.

Of the drugs used here five were also tested by Anton (1961) for ability to displace sulphaethylthiadiazole. His findings correspond with those reported here for sulphamethoxypyridazine in that phenylbutazone and sulphinpyrazone were good displacing agents, tolbutamide and salicylate were less active and probenecid was very weak.

Since the present work was completed Kunin (1965b) has reported the displacement of phenoxymethylpenicillin from human serum by a number of antagonists. Kunin used the dialysis method of Anton (1961) but with a relatively smaller buffer compartment. In Kunin's (1956b) dialysis system the displacing activity from human serum was in the order salicylate>sulphinpyrazone>tolbutamide>novobiocin>2:6 dihydroxybenzoate >phenylbutazone>probenecid. In the present study, on the other hand, the displacing activity from bovine serum albumin as measured by ultrafiltration was in the order 2:6 dihydroxybenzoate>salicylate>sulphinpyrazone>phenylbutazone>novobiocin>probenecid.

Is displacement from binding to plasma proteins *in vivo* likely to be of practical importance? The antagonist concentrations used in the present study are in the upper range of the concentrations attainable in plasma *in vivo*. However, if a substance is highly bound in plasma the displacement of only a small percentage of it from the proteins will bring about a significant increase in free drug concentration. Several instances of displacement from binding *in vivo* have been reported. In premature infants sulfisoxazole displaces bilirubin from binding to albumin and the resultant increase in free bilirubin concentration allows more bilirubin to cross the blood-brain barrier and so increases the incidence of kernicterus (Harris, Lucey & Maclean, 1958). Bilirubin may also be displaced by salicylate (Schmid, Diamond, Hammaker & Gundersen, 1965). Osorio (1963) found that salicylate displaced thyroxine from binding to albumin and to thyroxine-binding globulin *in vivo*. A number of drugs displace sulphonamides (Anton, 1961) and penicillins (Kunin, 1965a) from plasma proteins *in vivo* thus increasing the concentration of these drugs in the tissues. Experiments are proceeding to determine

whether displacing agents potentiate the action of antibiotics in the treatment of infections.

SUMMARY

1. The displacement of sulphamethoxypyridazine, phenoxymethylpenicillin and phenol red from binding to bovine serum albumin has been studied by ultrafiltration. Ten anionic drugs were used as displacing agents.

2. The curve relating the concentration of displacing agent to the percentage of bound drug displaced was linear over the mid-range and the curves for the different displacing agents were substantially parallel. These curves were used to compare the activities of the different displacing agents against each of the three bound drugs. The percentage of bound drug displaced by a particular concentration of displacing agent was independent of the total concentration of bound drug.

3. Ease of displacement was in the order sulphamethoxypyridazine>phenoxymethylpenicillin>phenol red.

4. The displacing agents used showed some specificity. The most effective antagonists of sulphamethoxypyridazine were phenylbutazone, sulphinpyrazone and tolbutamide; of phenoxymethylpenicillin: *p*-iodobenzoate, 2:6 dihydroxybenzoate and salicylate; of phenol red: novobiocin, *p*-iodobenzoate and phenylbutazone.

5. The nature of binding and of displacement are discussed in the light of the specificity shown by the displacing agents and the shape of the displacement curves.

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