# Enhancement of Antimicrobial Activity of Penicillins and Other Antibiotics in Human Serum by Competitive Serum Binding Inhibitors.* (29499)

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Serum binding of metabolites and chemotherapeutic agents is well known and has been extensively described(1-3). Attachment of antimicrobial agents to components of serum may profoundly affect their activity, delay their excretion and limit their distribution in tissues depending upon the extent and reversibility of binding(4,5). Excessive serum protein binding has rendered some otherwise effective drugs useless as chemotherapeutic agents and tends to restrict the usefulness of others(6,7). An investigation of the potential value of inhibitors of serum binding of penicillins and other antibiotics was stimulated by the advent of new penicillinase resistant penicillins which are strongly bound to serum(8), by the known competition between bilirubin and various sulfonamides for serum protein binding(3), and by the demonstration by Anton(9) that analogues of phenylbutazone could markedly alter the serum binding and pharmacologic behavior of sulfonamides.

A series of equilibrium dialysis experiments was performed employing C14 labeled penicillin G, V, and ancillin (diphenyl penicillin) to determine the extent of binding of these penicillins in serum of man and other animals and to screen potential inhibitors of this binding. The present report describes the effect of some of the most active of the nearly 250 compounds screened by this method in enhancing the antibacterial activity of penicillins and some other antibiotics in the presence of serum. Detailed reports of the quantitative dialysis studies and of studies of the effect of these agents on distribution and binding of penicillins *in vivo* are in preparation.

*Material and methods. Staphylococcus,* strain 209 P, kindly provided by Dr. Maxwell Finland, was employed in all experiments. It was maintained by serial passage in trypticase soy broth (TSB). Groups of 10 to 20 volunteers were bled 500 ml each. Sera were separated from cells, pooled, filtered and frozen at —20°C until used. Each antibiotic was serially, two-fold, diluted in TSB. Replicate 0.25 ml aliquots of each dilution were distributed in a series of 8 or more rows of tubes for each antibiotic. TSB or whole human serum in 0.5 ml volumes were then added to all tubes followed by 0.1 ml of saline (0.85% NaCl) or the drug to be tested for displacing activity or competitive binding. Tubes were then incubated at 37°C for 1 hour and 0.2 ml of a $1 \times 10^{-4}$ dilution of *Staphylococcus* 209 P in TSB containing 3% human O cells was then added to all tubes. The inoculum size varied from 2-6 $\times$ $10^4$ organisms per ml. Mixtures were incubated overnight at 37°C, and bacterial growth observed grossly and by darkening of erythrocytes. The TSB containing the inoculum included (unless stated otherwise) 68 mg per 100 ml of para-amino benzoic acid (PABA) which had been autoclaved at 15 lb for 15 minutes to yield a final concentration in culture tubes $1 \times 10^{-3}$ M. Controls to detect possible antimicrobial activity of binding inhibitors were included in each test, as described above, except that penicillin was not added.

*Results. Effect of human serum on antimicrobial activity of various antibiotics.* The effect of 50% human serum compared to TSB on the minimum inhibiting concentration of a series of penicillins, cephalothin and novobiocin is shown in Fig. 1. Differences noted between serum and TSB were readily reproducible; the values depicted are the means of 10 or more experiments with all but the 3 drugs listed at the bottom of the

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TABLE I. Minimum Molar Concentration (times $1 \times 10^{-4}$) of Displacing Agents Required to Enhance Antibacterial Activity of Various Penicillins and Cephalothin 2-Fold or Greater in Presence of 50% Human Serum.

| Displacing agent† | G* | V | An | Ox | Naf | Meth | Ceph |
|---|---|---|---|---|---|---|---|
| Sulfonamides | | | | | | | |
| sulfamethoxypyridazine | 10 | 5 | 2 | 5 | 5 | >10 | 10 |
| sulfisoxazole | 10 | 5 | 2 | 5 | 5 | >10 | >10 |
| N′ (5 ethyl, 1,3,4, thiadazol 2 yl) sulfanilamide | 10 | 2 | 2 | 5 | <1 | >10 | 10 |
| acetyl sulfaethylthiadazol | 10 | 5 | 2 | 5 | 5 | >10 | 10 |
| sulfisomidine | >10 | 5 | 2 | 5 | 5 | >10 | 10 |
| sulfadiazine | 10 | >10 | >10 | >10 | >10 | >10 | ND‡ |
| sulfapyradine | 10 | >10 | >10 | >10 | >10 | >10 | ND |
| Salicylates | | | | | | | |
| acetyl salicylic acid | < 1 | 10 | 10 | 10 | >10 | >10 | >10 |
| 3 phenyl salicylic acid | 10 | 5 | 2 | 5 | 10 | >10 | >10 |
| 5 bromo salicylic acid | 10 | 2 | 5 | 5 | 10 | >10 | >10 |
| 0 cresotinic acid | >10 | 5 | <1 | 5 | 10 | >10 | >10 |
| Penicillin "R" groups | | | | | | | |
| 5 methyl 3 phenyl isoxazole 4 carboxylic acid | >10 | 2 | 2 | 2 | < 1 | >10 | 10 |
| 2 ethoxy 1 naphthoic acid | 5 | 10 | 2 | 10 | 5 | >10 | >10 |
| Others | | | | | | | |
| d or l penicillamine | >10 | >10 | >10 | >10 | >10 | >10 | ND |
| antipyrine | >10 | >10 | >10 | >10 | >10 | >10 | ND |
| probenecid | >10 | >10 | >10 | >10 | >10 | >10 | ND |

* G = benzyl penicillin, V = phenoxymethyl penicillin, An = diphenyl penicillin, Ox = oxacillin, Naf = nafcillin, Meth = methicillin, Ceph = cephalothin.
† All tubes contained $1 \times 10^{-3}$ M para-amino benzoic acid.
‡ ND = Not done.

Figure which were tested 3 times. Similar studies with tetracycline and oxytetracycline revealed 4 and 3 tube reduction of activity, respectively, in the presence of 50% human serum. Addition of para-amino benzoic acid had no effect on these end points, and at the concentration employed PABA was not inhibitory to the test organism.

*Effect of displacing agents on the antibacterial activity of penicillins in the presence of PABA.* A summary of experiments to determine the effect of various concentrations of compounds, many of which were known to inhibit the binding of radiolabeled penicillin G, V and ancillin by human serum, on the antibacterial activity of a series of penicillins and cephalothin is presented in Table I. The minimum molar concentration of each drug which enhanced activity by one or more 2-fold dilution is shown. Drugs indicated by $>10 \times 10^{-4}$ M had no activity in this test. Only the first 5 sulfonamides listed were active. Sulfadiazine and sulfapyridine were virtually inactive in the radiolabelled drug experiments and in the experiments reported here; they are included to demonstrate that not all sulfonamides are active displacing agents. The lack of effect of these latter two

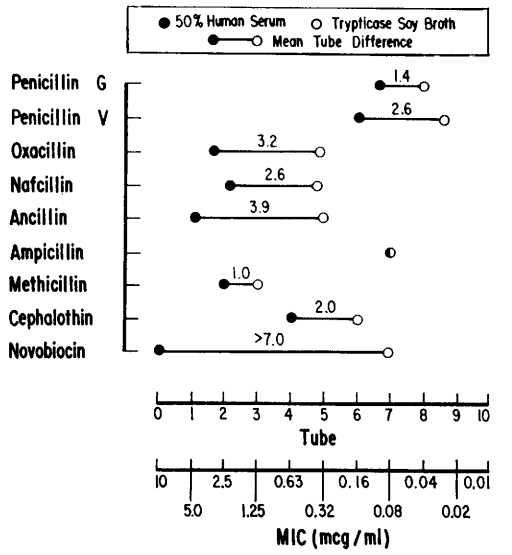


FIG. 1. Effect of 50% human serum and trypticase soy broth on minimal inhibiting concentrations of penicillins, cephalothin and novobiocin against *Staphylococcus* 209 P.

TABLE II. Effect of $1 \times 10^{-3}$ Molar Para-amino Benzoic Acid on Enhancement of Antimicrobial Activity of Penicillins in 50% Human Serum by $1 \times 10^{-3}$ Molar Sulfonamides and Other Drugs.

| Penicillin | G | | V | | Ancillin | | Oxacillin | | Nafcillin | |
|---|---|---|---|---|---|---|---|---|---|---|
| PABA, $1 \times 10^{-3}$ M | No | Yes | No | Yes | No | Yes | No | Yes | No | Yes |
| Sulfonamides | Fold increase in penicillin activity* | | | | | | | | | |
| sulfamethoxypyridazine | 4 | 2 | 4 | 2 | 2 | 0 | 8 | 4 | 4 | 2 |
| sulfisoxazole | 4 | 2 | 4 | 2 | 4 | 2 | 8 | 4 | 4 | 2 |
| N′ (5 ethyl, 1,3,4, thiadazol 2 yl) sulfonilamide | 2 | 0 | 4 | 2 | ND† | ND | 8 | 4 | ND | ND |
| acetyl sulfaethylthiadazol | 2 | 2 | 4 | 4 | 2 | 2 | 4 | 4 | 2 | 2 |
| sulfisomidine | 4 | 0 | 2 | 0 | 4 | 2 | 4 | 2 | 4 | 2 |
| sulfadiazine | 4 | 2 | 2 | 0 | 2 | 0 | 2 | 0 | 2 | 0 |
| sulfapyridine | 4 | 2 | 4 | 0 | 4 | 0 | 2 | 0 | 0 | 0 |
| Salicylates | | | | | | | | | | |
| acetyl salicylic acid | 2 | 2 | 2 | 2 | 2 | 2 | 4 | 4 | 2 | 2 |
| 5 bromosalicylic acid | 2 | 2 | 2 | 2 | 2 | 2 | 4 | 4 | 2 | 2 |
| Others | | | | | | | | | | |
| 5 methyl 3 phenyl isoxazole 4 carboxylic acid | 0 | 0 | 2 | 2 | ND | ND | 2 | 2 | ND | ND |
| antipyrine | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| sulfinpyrazone | 0 | 0 | 0 | 0 | 2 | 2 | 2 | 2 | 2 | 2 |

* Tests performed in replicate 2-fold dilution series. † ND = Not done.

drugs also served to demonstrate that the PABA added to the system was effective in abolishing the antibacterial effect of sulfonamide.

The salicylates and penicillin "R" groups (as carboxylic acids) demonstrated displacing activity similar to the sulfonamides. Drugs in the category "others" were included in these tests to determine whether compounds related to the nucleus of the penicillin molecule (penicillamine), or with antipyretic action (anti-pyrine), or employed to block tubular secretion of penicillin (probenecid) had any activity as displacing agents. None of these were active displacing agents in the current antibacterial or in the dialysis studies.

Agents found to be effective competitors for many of the penicillin analogues had no effect on methicillin activity in serum, and at best had very little effect on penicillin G. These 2 penicillins are known to be less extensively bound to serum proteins than other drugs tested (Fig. 1). Enhancement of antimicrobial activity greater than 2-fold was observed only with inhibitors added to serum containing oxacillin. Four-fold enhancement of oxacillin at a concentration of $5 \times 10^{-4}$ M was found with 5 methyl 3 phenyl isoxazole 4 carboxylic acid (the "R" group of this drug) and with N′ (5 ethyl 1,3,4, thiadazol 2 yl) sulfonilamide and acetylsulfaethylthiadazole.

*Effect of PABA on enhancement of activity of penicillins in serum by sulfonamides.* None of the sulfonamides tested at $1 \times 10^{-3}$ molar exhibited antibacterial activity against *Staphylococcus* 209 P in absence of the penicillins. A series of experiments summarized in Table II, however, indicates that when PABA was omitted from the medium sulfonamides at $1 \times 10^{-3}$ M exhibited augmentation of penicillin activity in serum beyond that thought to be due to serum binding inhibition. Augmented penicillin antibacterial activity reversible by PABA, could be demonstrated with sulfadiazine and sulfapyridine, both of which have little or no effect on serum binding of penicillins. Salicylates and other non-sulfonamide compounds tested did not augment the antibacterial activity of penicillins beyond their known effect on serum binding; and endpoints for the latter drugs were unaffected by the presence or absence of PABA.

*Effect of inhibitors of penicillin serum binding on activity of other antibiotics in serum.* All of the agents employed in the present study were tested to determine whether they would reverse the deleterious

effect of human serum on the antibacterial activity of novobiocin, tetracycline and oxytetracycline. No enhanced activity could be demonstrated.

*Discussion.* The current report is part of a series of studies designed to evaluate the effect of serum binding on the body distribution and antimicrobial activity of penicillins and related drugs and to develop methods to reverse such binding. Studies to be reported demonstrate that extensive serum binding may adversely effect the distribution of penicillins in the tissues of rabbits. The current data document well known observations of inhibition of antimicrobial activity of some penicillins by serum. Serum binding does not appear to be a major therapeutic problem with well established agents such as benzyl or phenoxymethyl penicillin (penicillin G, and V respectively) nor with such new synthetic analogues as methicillin and ampicillin which are only lightly bound to serum protein. It may be of some importance with more highly bound synthetic penicillins such as ancillin (diphenyl penicillin), oxacillin and nafcillin(8,10).

Screening of drugs which might displace penicillin from serum binding sites was conducted with radiolabelled penicillins in dialysis studies to permit rapid survey of many compounds and to avoid the complication of interpretation of results in bioassay systems when the test drug might also exhibit antibacterial activity. Similar methods have been used by Pressman and Siegel in studies of competitive binding in rabbit serum(11). Those drugs found to be active in the current study were also the most active in the isotope studies, and the inactive drugs reported here were also virtually inactive in the dialysis tests. Furthermore, the molar concentrations required to augment antibacterial activity are in the same range as found in the dialysis studies. The method employed in the current report is less sensitive than equilibrium dialysis for detecting displacing agents, but augmented antibacterial activity was clearly shown with the most active agents screened thus far.

Para-amino benzoic acid proved useful in distinguishing between the antimicrobial and the displacing effects of sulfonamides in augmentation of the activity of penicillins in serum. The concentration of PABA employed was well in excess of that reported by Janeway(12) to completely eliminate sulfonamide activity. An apparent synergistic effect of penicillin and sulfonamides in excess of displacing activity was observed in studies in which PABA was excluded from the medium. *Staphylococcus* 209 P was not sensitive to sulfonamides at $1 \times 10^{-3}$ molar, but when sulfonamides were added to very small amounts of penicillin in terminal dilution tubes they demonstrated enhanced antibacterial activity which could not be blocked by PABA. This was most clearly demonstrated with sulfadiazine and sulfapyridine, drugs with little or no displacing activity. Salicylates and other drugs tested did not show this effect.

The demonstration that antibacterial activity of novobiocin and tetracyclines in serum could not be augmented by the penicillin serum displacing agents indicates that these antibiotics are bound to serum at sites other than those which bind penicillins. Probenecid, although reported to be highly serum bound, did not displace penicillin. This is further evidence for the specificity of the binding site(s). Sulfonamides which exerted the strongest displacing effect are known to be much more highly bound to serum than those which had little activity(2,5). Salicylates are also highly bound to serum albumin (14).

The "R" group or radical which, when linked to 6 aminopenicillanic acid, defines each penicillin analogue, appears to be the most important determinant of binding. Thus, 5 methyl 3 phenyl isoxazole 4 carboxylic acid (the carboxylic acid of the "R" group of oxacillin) and 2 ethoxy 1 naphthoic acid, (the "R" group of nafcillin) were active displacing agents. Penicillamine, part of the drug nucleus, and 6 aminopenicillanic acid were poor displacing agents in dialysis experiments. This is not surprising since 6 amino penicillanic acid is present in all penicillins regardless of the extent to which they are bound to serum.

The concentrations of sulfonamides and

salicylates required for effective displacing activity are in the range of $2 \times 10^{-4}$ to $1 \times 10^{-3}$ molar. This is equivalent to 5.6-28.0 and 3.6-18.0 mg per 100 ml of sulfamethoxypyridazine and acetylsalicylic acid, respectively. The most effective concentrations are in the upper range achieved with customary chemotherapeutic doses of these drugs(15,16). It remains to be determined whether these or similar agents will prove useful as adjuncts to penicillin chemotherapy.

*Summary.* A series of compounds, many of which had been shown in dialysis studies to be effective in displacing penicillins from binding to human serum, have been tested for ability to enhance the antimicrobial activity of various penicillins and other antibiotics in the presence of 50% human serum. Enhanced antimicrobial activity in serum was shown in the case of sulfonamides to be only partially reversible by para-amino benzoic acid, suggesting that augmentation of penicillin activity was due to serum displacing effects as well as to the intrinsic antibacterial activity of sulfonamides. Evidence is presented that the "R" groups of penicillins are the most important determinants of binding to serum. The specificity of the penicillin binding sites was shown by the failure of displacing agents shown to be effective against the penicillin site(s), to effect binding of novobiocin and tetracyclines, and by the lack of effect of probenecid, known to be highly bound, to displace penicillins from serum.

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## Effect of Pyridoxine Deficiency on Rat Adrenal Phosphorylase.* (29500)

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Pyridoxal 5-phosphate, the prosthetic group of muscle polysaccharide phosphorylase, is essential for catalytic activity as well as for maintenance of the stable tetramer form of the enzyme(1,2). The phosphorylase molecule upon removal of pyridoxal 5-phosphate exhibits little enzymatic activity; however, this can be restored almost completely by recombining the apoenzyme with pyridoxal 5-phosphate(3). Illingworth *et al*(4) have shown that pyridoxine deficiency in the rat causes a reduction of total muscle phosphorylase but found no change in phosphorylase

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